ADVANCES IN Agronomy

Volume 90

Advances in Agronomy

Advances in Agronomy

Volume 90

Edited by

Donald L. Sparks

Department of Plant and Soil Sciences
University of Delaware
Newark, Delaware

Contents

PATHOGENS IN BIOSOLIDS

Ian L. Pepper, John P. Brooks and Charles P. Gerba

ADVANCES IN CROP WATER MANAGEMENT USING CAPACITIVE WATER SENSORS

A. Fares and V. Polyakov

Synchrotron Radiation Infrared Spectromicroscopy: A Noninvasive Chemical Probe for Monitoring Biogeochemical Processes

H.-Y. N. Holman and M. C. Martin

DEVELOPMENT AND TESTING OF "ON-FARM" SEED PRIMING

D. Harris

THERMODYNAMIC MODELING OF METAL ADSORPTION ONTO BACTERIAL CELL WALLS: CURRENT CHALLENGES

Jeremy B. Fein

ALFALFA WINTER HARDINESS: A RESEARCH RETROSPECTIVE AND INTEGRATED PERSPECTIVE

Yves Castonguay, Serge Laberge,
E. Charles Brummer and Jeffrey J. Volenec

PROJECTING YIELD AND UTILIZATION POTENTIAL OF SWITCHGRASS AS AN ENERGY CROP

Samuel B. McLaughlin, James R. Kiniry,
Charles M. Taliaferro and Daniel De La Torre Ugarte

This page intentionally left blank

Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

John P. Brooks (1), *Waste Management and Forage Research Unit, USDA ARS, Mississippi State, Mississippi 39762*

E. Charles Brummer (203), *Department of Agronomy, Iowa State University, Ames, Iowa 50011*

Yves Castonguay (203), *Soils and Crops Research and Development Center, Agriculture and Agri-Food Canada, Québec G1V 2J3, Canada*

A. Fares (43), *Natural Resources and Environmental Management Department, College of Tropical Agriculture and Human Resources, University of Hawaii-Manoa, Honolulu, Hawaii 96822*

Jeremy B. Fein (179), *Civil Engineering and Geological Sciences, University of Notre Dame, Notre Dame, Indiana 46556*

Charles P. Gerba (1), *Department of Soil, Water and Environmental Science, The University of Arizona, Tucson, Arizona 85706*

D. Harris (129), *CAZS Natural Resources, University of Wales, Bangor, Gwynedd LL57 2UW, United Kingdom*

H.-Y. N. Holman (79), *Ecology Department, Earth Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720; Virtual Institute for Microbial Stress and Survival, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720*

James R. Kiniry (267), *USDA Agricultural Research Service, Grassland, Soil and Water Research Laboratory, Temple, Texas 76502*

Serge Laberge (203), *Soils and Crops Research and Development Center, Agriculture and Agri-Food Canada, Québec G1V 2J3, Canada*

M. C. Martin (79), *Advanced Light Source Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720*

Samuel B. McLaughlin (267), *Bioenergy Feedstock Development Program, Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831*

Ian L. Pepper (1), *Environmental Research Laboratory, Department of Soil, Water and Environmental Science, The University of Arizona, Tucson, Arizona 85706*

V. Polyakov (43), *Natural Resources and Environmental Management Department, College of Tropical Agriculture and Human Resources, University of Hawaii-Manoa, Honolulu, Hawaii 96822*

Charles M. Taliaferro (267), *Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, Oklahoma 74078*

Daniel De La Torre Ugarte (267), *US Department of Energy, Agricultural Policy Analysis Center, University of Tennessee, Knoxville, Tennessee 37996*

Jeffrey J. Volenec (203), *Department of Agronomy, Purdue University, West Lafayette, Indiana 47907*

Preface

Volume 90 contains seven cutting-edge reviews that will be of interest to crop and soil scientists as well as other professionals and students working in the plant, soil, and environmental sciences. Chapter 1 is a timely and comprehensive review of pathogens in biosolids. Topics that are discussed include: a historic perspective and current outlook; pathogens of concern in class B biosolids; and pathogen transport and survival in soil, water, and air. Chapter 2 describes advances in crop water management using capacitive water sensors. Chapter 3 discusses the application of synchrotron-based infrared spectromicroscopy to the study of important biogeochemical reactions and processes in the environment. Chapter 4 covers the topic of "on-farm" seed priming as it relates to the production and management of various agronomic crops. Chapter 5 discusses research accomplishments and challenges related to modeling of metal adsorption on bacterial cell walls. Chapter 6 is a comprehensive review on alfalfa winter hardiness. Chapter 7 discusses the use of switchgrass as a bioenergy crop.

I appreciate the excellent contributions of the authors.

DONALD L. SPARKS
University of Delaware
Newark, Delaware

Pathogens in Biosolids

Ian L. Pepper,[1] John P. Brooks[2] and Charles P. Gerba[3]

[1]Environmental Research Laboratory,
Department of Soil, Water and Environmental Science,
The University of Arizona, Tucson, Arizona 85706
[2]Waste Management and Forage Research Unit,
USDA ARS, Mississippi State, Mississippi 39762
[3]Department of Soil, Water and Environmental Science,
The University of Arizona, Tucson, Arizona 85706

The world population of 6.8 billion people all produce sewage. In the developed world most of this is treated by the activated sludge process, which results in large volumes of sludge or biosolids being produced (NRC, 2002). This results in millions of tons of biosolids produced each year in the United States, which must either be disposed of or recycled in some manner. Land application has been seen as the most economical

1

and beneficial way of handling biosolids. Biosolids that result from municipal wastewater treatment processes contain organic matter and nutrients that, when properly treated and applied to farmland, can improve the productivity of soils or enhance revegetation of disturbed ecosystems. However, besides the documented benefits of land application, there are also potential hazards, which have caused the public response to the practice to be mixed. Here we review one of the potential hazards associated with biosolids and its land application, namely human pathogens associated with biosolids.

I.   BIOSOLIDS: A HISTORICAL PERSPECTIVE AND CURRENT OUTLOOK

In the United States, land application of municipal wastewater and biosolids has been practiced for its beneficial use and for disposal purposes since the advent of modern wastewater treatment about 160 years ago (NRC, 1996). In Britain, during the 1850s, "sewage farms" were established to dispose of untreated sewage. By 1875, about 50 farms were utilizing land treatment in England, as well as many other major cities in Europe. In the United States, sewage farms were established by about 1900. At this time, primary sedimentation and secondary biological treatment was introduced as a rudimentary form of wastewater treatment, and land application of "sludges" began. It is interesting to note that prior to modern activated sludge wastewater treatment, "sludge" per se did not exist. As early as 1907, municipal sludge in Ohio was used as a fertilizer (NRC, 1996). Early on land application was carried out with little regard to pollution, with maximum rates of sludge applied to minimize the costs of sludge disposal.

Since the early 1970s, more emphasis has been placed on applying sludge to cropland at an agronomic rate (Hinesly *et al.*, 1972). In the 1970s and 80s, many studies were undertaken to investigate the potential benefits and hazards of land application, in both the United States and Europe. Ultimately in 1993, Federal regulations were established via the "Part 503 Sludge Rule." This document—"The Standards for the Use and Disposal of Sewage Sludge"—(EPA, 1993) was designed to "adequately protect human health and the environment from any reasonably anticipated adverse effect of pollutants." As part of these regulations, two classes of treatment resulted in "Class A and Class B" biosolids, with different restrictions for land applications, based on the level of treatment.

Land application has increased since restrictions were placed on "ocean dumping disposal." Sixty percent of all biosolids are land applied in the United States, with most land application in the United States utilizing Class B biosolids (NRC, 2002). However, due to public concern over potential hazards, in some areas of the United States, land application of Class B biosolids has been banned. This is particularly true in California, where in many areas Class A land application has replaced Class B land applications.

II.   THE NATURE OF WASTEWATER (SEWAGE)

Domestic wastewater or sewage is a combination of human feces, urine, and graywater. Graywater results from washing, bathing, and meal preparation. Sewage sludge is defined in the Part 503 rule as the solid, semisolid, or liquid residue generated during the treatment of domestic sewage in a wastewater treatment plant (Box 1). The term biosolids is not used in the Part 503 rule, but EPA (1995) defines biosolids as "the primarily organic solid product yielded by municipal wastewater treatment processes that can be beneficially recycled" as a soil amendment. The term biosolids has been controversial because of the perception that it was created to improve the image of sewage sludge in a public-relations campaign by the sewage industry. Here, we use the term biosolids to imply treatment of sewage sludge to meet the land-application standards in the Part 503 rule. This definition was provided by the National Research Committee—"Biosolids applied to land: Advancing standards and practices" (2002).

Box 1
Definitions

Sewage sludge: The solid, semisolid, or liquid residue generated during the treatment of domestic sewage in a treatment works.
Biosolids:

- EPA's definition: The primarily organic solid product yielded by municipal wastewater treatment processes that can be beneficially recycled (whether or not they are currently being recycled).
- NRC, 2002 committee's definition: Sewage sludge that has been treated to meet the land-application standards in the Part 503 rule or any other equivalent land-application standards or practices.

It is estimated that approximately 5.6 million dry tons of sewage sludge are used or disposed of annually in the United States, of which approximately 60% are used for land application (NRC, 2002). In some states, such as Arizona, 95% of the biosolids are land applied. However, EPA estimates that only approximately 0.1% of available agricultural land in the United States is treated with biosolids (NRC, 2002).

Biosolids are applied to agricultural and nonagricultural lands as soil amendment because they can improve the chemical and physical properties of soils, and because they contain nutrients for plant growth. Land application on agricultural land is utilized to grow food crops, such as corn or wheat, and nonfood crops such as cotton. Nonagricultural land application includes forests, rangelands, public parks, golf courses, and cemeteries. Biosolids are also used to aid revegetation of severely disturbed lands, such as mine tailings or strip mine areas.

III.   WASTEWATER (SEWAGE) TREATMENT

Figure 1 provides a simplified schematic of how biosolids are produced as a result of wastewater treatment. Biosolids are a combination of primary sludge and secondary sludge, produced during the activated sludge process. Primary sludge results from the settling of solids as they enter a sewage treatment plant. Secondary sludge results from the conversion of soluble organic matter in the sewage to bacterial biomass. These two types of sludge are then combined and must be treated before land application. The final product is known as biosolids.

A.   Class A Versus Class B Biosolids

Biosolids are divided into two classes on the basis of pathogen content: Class A and Class B (Box 2). In essence, a higher level of treatment results in Class A biosolids, which has no detectable levels of pathogens. In contrast Class B biosolids, the result of a lower level of treatment, normally contain bacterial, parasitic, and viral pathogens (Box 2).

A summary of Class A and B pathogen reduction requirements are shown in Box 3. Processes to significantly reduce pathogens (PSRP) are shown in Box 4. PSRPs are the treatment alternatives for Class B status. Processes to further reduce pathogens (PFRP) are shown in Box 5. To meet Class A requirements with respect to pathogens, there are six alternative treatments available, including treatment with any PFRP. In addition to one of the six requirements, the requirements of Box 2 must also be met.

Domestic wastewater   Commercial wastewater   Industrial wastewater

Pretreatment[a]

Municipal wastewater

Conventional wastewater treatment
• Screening and grit removal
• Primary, secondary, and tertiary treatment

Screenings and grit
to landfill

Effluent to surface
or groundwater

Sewage sludge

Treatment

Stabilization
   -Alkaline stabilization
   -Anaerobic digestion
   -Aerobic digestion
   -Composting
   -Heat drying and
    pelletizing
   -Other

Dewatering[b]
   -Air drying
   -Vacuum filters
   -Centrifuges
   -Belt filter presses
   -Plate and frame

Disposal
• Incineration
• Surface disposal
• Landfill

Land application/
Public distribution

Figure 1   Simplified scheme of biosolids production (From NRC, 2002). [a]Required by federal and state agencies; [b]prior to dewatering, sewage sludge is conditioned and thickened by adding chemicals (e.g., ferric chloride, lime, or polymers).

Class A biosolids are treated to reduce the presence of pathogens to below detectable levels and can be used without any pathogen-related restrictions at an application site. Class A biosolids can also be bagged and sold to the public as a fertilizer. Class B biosolids are also treated to reduce pathogens, but still contain detectable levels. Class B biosolids have site restrictions to minimize the potential for human and animal exposure until environmental

---

Box 2
Part 503 Pathogen Density Limits Adapted from US EPA 2000

Part 503 pathogen density limits

Pathogen or indicator	Standard density limits (dry wt.)
Class A	
Salmonella	3 MPN per 4 g total solids
Fecal coliforms	<1000 MPN per g
Enteric viruses	<1 PFU per 4 g total solids
Viable helminth ova	<1 per 4 g total solids
Class B	
Fecal coliform density	<2,000,000 MPN per g total solids

---

factors such as heat, sunlight, or dessication have reduced pathogens further. Class B biosolids cannot be sold, given away, or used at sites with public use. The overall concept here is that Class B biosolids plus site restrictions are equivalent to Class A biosolids with respect to the potential hazard of pathogens. The principal pathogens of concern in Class B biosolids are illustrated in Box 6.

IV. REMOVAL OF PATHOGENS BY SEWAGE TREATMENT PROCESSES

Compared with other biological treatment methods (i.e., trickling filters), activated sludge is relatively efficient in reducing the number of pathogens in raw wastewater. Primary sedimentation is more effective for the removal of the larger pathogens, such as helminth eggs, but solid-associated bacteria and even viruses are also removed. The greatest removal probably occurs by adsorption or entrapment of the organisms within the biological floc that forms. The ability of activated sludge to remove viruses is related to the ability to remove solids. This is because viruses tend to be solid associated and are removed along with the floc. Activated sludge typically removes 90% of the enteric bacteria and from 80 to 99% of the enteroviruses and rotaviruses (Rao *et al.*, 1986) (See also Table I). Ninety percent of *Giardia* and *Cryptosporidium* can also be removed (Rose and Carnahan, 1992), being largely concentrated in the sludge. Because of their large size, helminth eggs are effectively removed by sedimentation and are rarely found in sewage effluent in the United States, although they may be detected in the sludge.

Box 3
Summary of Class A and Class B Pathogen Reduction Requirements

Class A

In addition to meeting the requirements in one of the six alternatives listed below, fecal coliform or *Salmonella* sp. bacteria levels must meet specific density requirements at the time of biosolids use or disposal, or when prepared for sale or give away.

Alternative 1: Thermally treated biosolids
Use one of four time-temperature regiments.

Alternative 2: Biosolids treated in a high pH-high temperature process
Specifies pH, temperature, and air-drying requirements.

Alternative 3: For biosolids treated in other processes
Demonstrate that the process can reduce enteric viruses and viable helminth ova. Maintain operating conditions used in the demonstration.

Alternative 4: Biosolids treated in unknown processes
Demonstration of the process is unnecessary. Instead, test for pathogens—*Salmonella* sp. or fecal coliform bacteria, enteric viruses, and viable helminth ova—at the time the biosolids are used or disposed of are prepared for sale or give away.

Alternative 5: Use of PFRP
Biosolids are treated in one of the Processes to further reduce pathogens (PFRP).

Alternative 6: Use of a process equivalent to PFRP
Biosolids are treated in a process equivalent to one of the PFRPs, as determined by the permitting authority.

Class B

The requirements in one of the three alternatives below must be met:

Alternative 1: Monitoring of indicator organisms
Test for fecal coliform density as an indicator for all pathogens at the time of biosolids use or disposal.

Alternative 2: Use of PSRP
Biosolids are treated in one of the processes to significantly reduce pathogens (PSRP).

Alternative 3: Use of processes equivalent to PSRP
Biosolids are treated in a process equivalent to one of the PSRPs, as determined by the permitting authority.

Source: EPA, 1994.

Although the removal of the enteric pathogens may seem large, it is important to note that initial concentrations are also large (i.e., the concentration of all enteric viruses in 1 liter of raw sewage may be as high as 100,000 in some parts of the world) (Buras, 1974).

Box 4
Processes to Significantly Reduce Pathogens (PSRPs)

1. Aerobic digestion

Biosolids are agitated with air or oxygen to maintain aerobic conditions for a specific mean cell residence time at a specific temperature. Values for the mean cell residence time and temperature shall be between 40 days at 20°C and 60 days at 15°C.

2. Air drying

Biosolids are dried on sand beds or on paved or unpaved basins. The biosolids dry for a minimum of 3 months. During 2 of the 3 months, the ambient average daily temperature is above 0°C.

3. Anaerobic digestion

Biosolids are treated in the absence of air for a specific mean cell residence time at a specific temperature. Values for the mean cell residence time and temperature shall be between 15 days at 35°C to 55°C and 60 days at 20°C.

4. Composting

Using either the within-vessel, static aerated pile, or windrow composting methods, the temperature of the biosolids is raised to 40°C or higher and maintained for 5 days. For 4 h during the 5-day period, the temperature in the compost pile exceeds 55°C.

5. Lime stabilization

Sufficient lime is added to the biosolids to raise the pH of the biosolids to 12 after 2 h of contact.

Source: EPA, 1994.

V. PATHOGENS OF CONCERN IN CLASS B BIOSOLIDS

A. BACTERIA

1. *Salmonella*

Salmonella is a very large group of bacteria comprising more than 2400 known serotypes. All these serotypes are pathogenic to humans and can cause a range of symptoms from mild gastroenteritis to severe illness or even death. *Salmonella* are capable of infecting a large variety of both cold- and warm-blooded animals. Typhoid fever, caused by *S. typhi*, is an enteric fever that occurs only in humans and primates. In the United States, salmonellosis is primarily due to foodborne transmission since the bacteria are found in beef and poultry products and are capable of growing in foods. The pathogens produce a toxin that causes fever, nausea, and diarrhea, and

---

Box 5
Processes to Further Reduce Pathogens (PFRPs)

1. Composting

Using either the within-vessel composting method or the static aerated pile composting method, the temperature of the biosolids is maintained at 55°C or higher for 3 days.

Using the windrow composting method, the temperature of the biosolids is maintained at 55°C or higher for 15 days or longer. During the period when the compost is maintained at 55°C or higher, the windrow is turned a minimum of five times.

2. Heat drying

Biosolids are dried by direct or indirect contact with hot gases to reduce the moisture content of the biosolids to 10% or lower. Either the temperature of the biosolids particles exceeds 80°C or the wet bulb temperature of the gas in contact with the biosolids as the biosolids leave the dryer exceeds 80°C.

3. Heat treatment

Liquid biosolids are heated to a temperature of 180°C or higher for 30 min.

4. Thermophilic Aerobic Digestion

Liquid biosolids are agitated with air or oxygen to maintain aerobic conditions, and the mean cell residence time of the biosolids is 10 days at 55°C to 60°C.

5. β-Ray irradiation

Biosolids are irradiated with β-rays from an accelerator at dosages of at least 1.0 megarad at room temperature (ca. 20°C).

6. γ-Ray irradiation

Biosolids are irradiated with γ-rays from certain isotopes, such as Cobalt 60 and Cesium 137, at room temperature (ca. 20°C).

7. Pasteurization

The temperature of the biosolids is maintained at 70°C or higher for 30 min or longer.

Source: EPA, 1994.

---

may be fatal if not properly treated (Rusin *et al.*, 2000). The number of *Salmonella* routinely found in Class B biosolids is approximately 1–400 g^{-1} dry biosolids (Zaleski *et al.*, 2005a). Because they have the potential to grow in biosolids, they are the bacteria of greatest concern in biosolids (Zaleski *et al.*, 2005b).

2. *Shigella*

Shigella is closely related to *Escherichia coli*. Four species have been described: *S. dysentariae*; *S. flexneri*; *S. boydii*; and *S. sonnei*. *S. dysentariae*

Box 6
Principal Pathogens of Concern in Class B Biosolids

Bacteria   **Protozoa**
Salmonella sp.   *Cryptosporidium*
Shigella sp.   *Entamoeba histolytica*
Yersinia   *Giardia lamblia*
Vibrio cholerae   *Balantidium coli*
Campylobacter jejuni   *Toxoplasma gondii*
Escherichia coli

Enteric viruses   **Helminth worms**
Hepatitis A virus   *Ascaris lumbricoides*
Adenovirus   *Ascaris suum*
Norovirus   *Trichuris trichirua*
Sapporovirus   *Toxocara canis*
Rotavirus   *Taenia saginata*
Enteroviruses   *Taenia solium*
• Polio viruses   *Necator americanus*
• Coxsackie viruses   *Hymenolepis nana*
• Echoviruses
• Enteroviruses 68–91
Reoviruses
Astroviruses
Hepatitis E virus
Picobirnavirus

---

causes the most severe disease and *S. sonnei* causes the mildest symptoms. Fortunately, *S. sonnei* is the serotype most often found in the United States (Lee *et al.*, 1991). The only source of the organism is believed to be of human origin. The organism is often found in water polluted with human sewage and is transmitted by the fecal–oral route. Surveillance data from the Centers for Disease Control and Prevention (CDC) between 1972 and 1985 showed that *Shigella* was the second most common cause of waterborne disease outbreaks of known cases, following *Giardia lamblia* (a parasite). An estimated 300,000 cases of shigellosis occur annually in the United States. *Shigella* is also associated with certain foods such as salads, raw vegetables, milk and dairy products, and poultry. After an onset time of 12–50 h, symptoms of abdominal pain, cramps, and diarrhea appear. However, most cases of shigellosis are the result of person-to-person transmission through the fecal–oral route, due to its relatively low infectious dose. *Shigella* spp. were shown to have a half-time die-off rate in fresh water at 9.5–12.5°C of 22.4–26.8 h (McFeters *et al.*, 1974). In well water, 50% of *S. flexneri* cells die off in 26.8 h (Gerba *et al.*, 1975). It occurs at concentrations much lower than other enteric bacteria pathogens in raw sewage sludge

Table I
Pathogen Removal During Sewage Treatment

	Enteric viruses	Salmonella	Giardia	Crypto-sporidium
Concentration in raw sewage (number per liter) Removal during Primary treatment[a]	10^5–10^6	5000–80,000	9000–200,000	1–3960
Percent removal	50–98.3	95.8–99.8	27–64	0.7
Number remaining (l^{-1}) Secondary treatment[b]	1700–500,000	160–3360	72,000–146,000	
Percent removal	53–99.92	98.65–99.996	45–96.7	
Number remaining (l^{-1}) Secondary treatment[c]	80–470,000	3–1075	6480–109,500	
Percent removal	99.983–99.999,999,8	99.99–99.999,999,995	98.5–99.999,95	2.7[d]
Number remaining (l^{-1})	0.007–170	0.000,004–7	0.099–2951	

[a]Primary sedimentation and disinfection.
[b]Primary sedimentation, trickling filter or activated sludge, and disinfection.
[c]Primary sedimentation, trickling filter or activated sludge, disinfection, coagulation, filtration, and disinfection.
[d]Filtration only.
Adapted from Yates (1994); Robertson *et al.* (1995); Modore *et al.* (1987), *Environmental Microbiology*
© Academic Press, San Diego, CA.

(Straub *et al.*, 1993b). Since *Shigella* do not survive well in the environment or after treatment of biosolids, they are unlikely to be a significant problem in biosolids.

3. *Escherichia coli*

Escherichia coli is found in the gastrointestinal tract of all warm-blooded animals and is usually considered a harmless commensal organism. However, several strains are capable of causing gastroenteritis; these are referred to as enterotoxigenic (ETEC); enteropathogenic (EPEC); enteroinvasive (EIEC); or enterohemorrhagic (EHEC) *E. coli*. All of these types are spread by the fecal–oral route of transmission.

Several types of *E. coli* are pathogenic to human. Enterohemorrhagic *E. coli* of the serotype 0157:H7 has been of the greatest concern in the United States. Exposure to contaminated drinking water, recreational water, and food has resulted in numerous outbreaks of diarrhea and, in some cases, mortality in young children because of hemolytic uremia syndrome. Exposure to both human and animal wastes has been associated with most outbreaks, as cattle are the most significant source of exposure (Rice, 1999). This strain of *E. coli* produces a toxin, which damages the kidneys and can be life threatening. *E. coli* 0157:H7 has been found in domestic wastewater and has been detected in biosolids (Lytle *et al.*, 1999). Sahlstrom *et al.* (2004) reported detecting it in 2% of the raw sewage sludge samples in Sweden and 0% of 69 samples of treated sludge (i.e., composting, thermophilic, and mesophilic digestion). Risks of infection from land-applied animal manures probably represent the greatest risk (Strachan *et al.,* 2002). Little data is available on the concentration and potential for growth of this pathogen in treated biosolids in the United States.

4. *Campylobacter*

Campylobacter jejuni is a gram-negative curved rod. It is relatively fragile and sensitive to environmental stress, however, it is the leading cause of bacterial diarrheal illness in the United States. It is often isolated from healthy cattle, chickens, birds, and even flies, and as a result, can be isolated from rivers and streams. Food is the major source of infection. *C. jejuni* infections cause diarrhea with fever, abdominal pain, nausea, headache, and muscle pain. The illness usually occurs 2–5 days after the ingestion of the contaminated food or water. Illness generally lasts 7–10 days, but relapses are not uncommon.

Very few studies have been performed on the occurrence of *Campylobacter* in sewage sludge. Jones *et al.* (1990) found number of *Campylobacter* as high as $42,000 \, ml^{-1}$ of raw sewage sludge, but were almost undetectable in anaerobically digested sludge before land application (8% of samples positive). Survival experiments suggested rapid inactivation of the *Campylobacter* in digested sludge. Stampi *et al.* (1999) reported concentrations ranging from 278 to $1403 \, MPN \, g^{-1}$ dry weight in primary sewage. However, *Campylobacter* was not detected in anaerobically digested sludges. Sahlstrom *et al.* (2004) reported isolating *C. jejuni* in 20% of raw sewage sludge samples and only in 2 of 69 treated samples (digested or composted). Other studies have also demonstrated that *Campylobacters* in sludge are eliminated by digestion processes (Stampi *et al.*, 1999; Stelzer *et al.*, 1991). In contrast, Horan *et al.* (2004) reported minimal reduction (0.36 log) in laboratory experiments, focusing on mesophilic digestion of raw sludge spiked with *C. jejuni*.

However, the authors noted spiked *C. jejuni* in the treated biosolids. *Arcobacter* spp. are a genus belong to the Campylobacteraceace that have the ability to grow at 15°C. This is a distinctive feature that differentiates them from the *Campylobacter* spp. While their role in human disease is unclear, intestinal outbreaks of disease have been associated with *Arobacter butzleri* (Lehner *et al.*, 2005). They are present in large numbers in raw sewage sludge and survive anaerobic digestion (7649 g per dry weight) (Stampi *et al.*, 1999).

5. *Yersinia*

Yersinia enterocolitica and *Y. pseudotuberculosis* are small rod-shaped gram-negative bacteria. Both organisms have often been isolated from animals such as pigs, birds, beavers, cats, and dogs. Only *Y. enterocolitica* has been detected in environmental and food sources such as ponds, lakes, meats, ice cream, and milk. To date, no foodborne outbreaks caused by *Y. pseudotuberculosis* have been reported in the United States, but human infections transmitted via contaminated water and foods have been reported in Japan. Food, especially pork products, is the major source of *Y. enterocolitica* infections in the United States, although waterborne outbreaks have also occurred (Bhaduri *et al.*, 2005). These infections are far less common than *Salmonella* and *Campylobacter* in the United States.

Symptoms usually begin 24–48 h after ingestion of contaminated food or drink. Yersiniosis is frequently characterized by such symptoms as gastroenteritis with diarrhea and/or vomiting. However, fever and abdominal pain are the hallmark symptoms. Yersinia infections mimic appendicitis, but the bacteria may also cause infections of other sites such as wounds, joints, and the urinary tract (Rusin *et al.*, 2000).

Environmental transmission of *Y. entreocolitica* appears to be uncommon, although several waterborne outbreaks have been suspected (Percival *et al.*, 2004). It has been detected in raw, digested, and dewatered biosolids (Straub *et al.*, 1993b), but little is know about its occurrence and survival in the environment.

6. Other Potential Bacterial Pathogens

a. **Listeria montocytogenes.** *L. montocytogenes* is primarily a foodborne pathogen that causes an invasive disease in immunocompromised people. It has a predilection for pregnant women and potentially lethal

consequences for the fetus and the newborn. Animals are also infected by these organisms. *L. montocytogenes* has been detected frequently in sewage sludge and in activated and anaerobically digested biosolids (DeLuca *et al.*, 1998; Watkins and Sleath, 1981). For that reason, DeLuca *et al.* (1998) suggested that biosolids should not be applied to vegetable crops. Crop contamination was observed in Iraq, where sewage-sludge cake was applied (Al-Ghazali and Al-Azawi, 1990). A risk-assessment model is available to evaluate the health risks associated with *L. montocytogenes* in contaminated food (Lindquist and Westoo, 2000).

b. Helicobacter pylori.   *H. pylori* is a major cause of stomach ulcers in humans and is associated with an increased risk of stomach cancer. Epidemiological evidence indicates that contaminated water and uncooked foods, particularly vegetables irrigated with untreated wastewater, are associated with increased risk of infection (Brown, 2000). *H. pylori* has been detected in wastewater, but current methods are difficult to apply to environmental samples and no studies on its occurrence in biosolids have yet been reported.

c. Legionella *spp*.   *Legionella* spp. are associated with a potentially life-threatening respiratory illness, often more commonly associated with the elderly or immunocompromised. *Legionella* is also associated with a milder fever and flulike illness, called Pontiac Fever. Outbreaks usually occur following growth of the organism in cooling towers of buildings or thermally heated water. However, outbreaks also have been associated with composted potting mixes (Okazaki *et al.,* 1998). An outbreak of Pontiac Fever was reported among sewage treatment-plant workers, repairing a decanter for sewage sludge concentration (Gregersen *et al.,* 1999). Positive antibody titers to *L. pneumophilia* were found in all the ill workers, and high concentrations were isolated from biosolids. *Legionella* has also been detected in aerosols at sewage treatment plants (Stampi *et al.,* 2000). *Legionella* spp. grow at temperatures of $40\,^{\circ}\mathrm{C}$, and survival at higher temperatures is also possible.

d. Staphylococcus aureus.   Speculation has arisen about the possibility of *S. aureus* illness due to land-applied biosolids. Although not always considered normal human microflora, *S. aureus* is nonetheless found on the skin and within the nasopharynx of a large number of people (McGinley *et al.*, 1988; Noble, 1998; Voss, 1975; Welbourn *et al.*, 1976). Some skin conditions associated with this bacteria include atopic dermatitis, a superficial inflammation of the skin (Nishijima *et al.*, 1995). It is uncertain whether *S. aureus* has a specific pathogen role in atopic dermatitis or its presence represents an opportunistic colonization at a site rendered more susceptible by an underlying condition, thus, complicating the clinical management

of these conditions. Eczema is another inflammatory skin condition that may have a *S. aureus* link. Eczema is characterized by redness, itching, and oozing lesions that can become scaly, crusted, or hardened. Increased severity and spreading of the condition has been associated with a cytotoxic effect of antibacterial antibody and complement reacting with bacterial antigens on skin cells (Welbourn *et al.*, 1976).

The incidence of *S. aureus* in sewage was documented by Rusin *et al.* (2003). However, that study also provided evidence for the absence of *S. aureus* in biosolids. Specifically, 23 different biosolid samples (7 Class A and 16 Class B) were collected from 15 sites across the United States. Analysis for *S. aureus* demonstrated that although *S. aureus* was found in raw sewage, it was never found in biosolids. Analyses of 37 air samples were also negative for *S. aureus*. These results suggest that biosolids are not a likely source of *S. aureus* human exposure or infection.

B. Enteric Viruses

Many human viruses may infect the gastrointestinal tract and be excreted in the feces into the environment (Table II). It has been estimated that an individual with an enteric viral infection may excrete 10^{11} viral particles per gram of feces. Once in the environment, viruses can reach water supplies, recreational waters, crops, and shellfish through sewage, land runoff, solid waste landfills, and septic tanks.

Diseases caused by enteric viruses range from trivial to severe, or even fatal. The viruses that are detected most often in polluted water are the

Table II
Human Enteric Viruses

Enteroviruses
    Poliovirus
    Coxsackie virus
    Echoviruses
    Enteroviruses (68–91)
Hepatitis A virus
Reoviruses
Rotaviruses
Adenoviruses
Astroviruses
Torovirus
    Hepatitis E virus
    Norovirus
    Saroprovirus

enteroviruses, however, this may be due to its ease of detection via animal cell culture. However, several studies have shown that adenoviruses (Hurst *et al.*, 1988), rotaviruses, and hepatitis A virus (HAV) (Moore *et al.*, 1993) can also be found in polluted water.

1. Adenoviruses

Adenoviruses are one of the most common and persistent viruses detected in wastewater (Enriquez *et al.*, 1995). Enteric adenoviruses have been detected in Class B biosolids (Sabalos, 1998), and adenovirus type 40 has been detected in anaerobically digested biosolids. Some adenoviruses cause primarily respiratory diseases and others appear to be only enteric pathogens. They are a common cause of diarrhea and respiratory infections in children. In immunosuppressed cancer patients, enteric adenoviruses cause serious infections, resulting in case fatalities of up to 50% (Gerba *et al.*, 1996). Adenoviruses have been transmitted by recreational and drinking waters (Kukkula *et al.*, 1997; Papapetropoulou and Vantarakis, 1998).

2. Hepatitis A and E Viruses

These viruses are now classified as two distinct groups of picornaviruses (small RNA viruses). Hepatitis E has caused major waterborne-disease outbreaks in developing countries but is not believed to be a serious problem in the Untied States. It has been reported to grow in cell culture (Wei *et al.*, 2000). Hepatitis A has long been known to be transmitted by food and water, but no work has been done on its occurrence in biosolids. Cell-culture methods are available for its growth in the laboratory and detection in the environment. It is very stable at high temperatures (Croci *et al.*, 1999) and has prolonged survival in the environment (Enriquez *et al.*, 1995). Hepatitis A virus has been detected by polymerase chain reaction (PCR) in biosolids, but the effectiveness of various treatments has not been studied (Graff *et al.*, 1993). There have been no reported studies on the occurrence of hepatitis E virus in biosolids, although they have been detected in swine manure slurry in the United States (Kasorndorkbua *et al.*, 2005).

3. Astroviruses and Rotaviruses

Astroviruses are a cause of gastroenteritis primarily in children and have been associated with foodborne and waterborne outbreaks. They have been detected in water, wastewater, and biosolids (Chapron *et al.*, 2000).

Rotaviruses are a leading cause of gastroenteritis in children and a major cause of hospitalization of children in the United States (Gerba *et al.*, 1996). Rotaviruses are responsible for waterborne and foodborne outbreaks in the United States. They have been detected in wastewater, but few data are available on their occurrence in biosolids (Hejkal *et al.*, 1984). Both astroviruses and rotaviruses can be grown in cell culture.

4. Calicivirus

Caliciviruses infect both humans and animals, but no evidence suggests that they infect across species. Human caliciviruses have been divided into two genera—the Noro viruses and the Sapporo viruses (Green *et al.*, 2000). These viruses are believed to be a major cause of viral gastroenteritis (Deneen *et al.*, 2000; Monroe *et al.*, 2000) and are common causes of foodborne and waterborne disease. Little is known about the occurrence and environmental fate of these viruses because they cannot be grown in cell culture. Methods, using PCR, are available for their detection in environmental samples, but a viability assay is not available (Huang *et al.*, 2000). Feline caliciviruses (FCV) and a primate calicivirus (PAN-1) can be grown in cell culture and have been used as models for human calicivirus survival and removal by water-treatment processes (Dawson *et al.*, 1993).

5. Enteroviruses

These are members of the family Picornaviridae and include poliovirus; Coxsackie virus; echovirus, and enteroviruses 69–91. The most common form of transmission include the fecal–oral and respiratory routes of infection. They are responsible for a wide variety of illnesses in humans, including meningitis, myocarditis, febrile illness, paralysis, respiratory infections, certain types of diabetes, and eye and skin infections. Because most are easily grown in cell culture, more is known about the occurrence and fate of these viruses in the environment. Poliovirus type 1 is perhaps the most studied enteric virus because it grows well in cell culture and a vaccine strain is available, which reduces biohazard concerns. Enteroviruses may survive for many weeks in the environment and are commonly isolated from wastewater and raw biosolids. The methods recommended for virus detection in biosolids in the 503 regulations are largely designed to detect enteroviruses. In general, enteroviruses are reduced by at least 90% during aerobic or anaerobic sludge digestion and other Class B processes. Class A processes will reduce them to below detection level in 4-g dry solids (Straub *et al.*, 1993b).

6.   Other Viruses of Concern

Because of advances in molecular biology, the number of viruses detected in water and wastewater continues to increase almost on a yearly basis. In many cases, the association with a particular disease or their potential for environmental transmission remains unclear. For example, JC virus, associated with mental illness in AIDS patients, is detectable in wastewater by molecular methods (Bofill-Mas *et al.*, 2003). Other viruses found in wastewater, such as circoviruses, have not yet been clearly shown to be associated with any particular illness (Biagini, 2004).

Blood borne and respiratory viruses can also be detected in wastewater and biosolids. How easily these viruses can be transmitted by wastewater is unclear. An epidemiological association between past hepatitis B infection and sewage workers has been demonstrated in at least one study (Arvanitidou *et al.*, 2004). Ansari *et al.* (1992) failed to detected the human immunodeficiency virus (HIV) RNA using reverse transcriptase PCR (RT-PCR) in several samples of raw sludge from an oxidation ditch, but was able to detect it in raw wastewater. It appears to be rapidly inactivated in the sewage and has not been detected in treated wastewater (Moore, 1993). Severe acute respiratory illness (SARS) virus is excreted in large numbers in the feces of infected individuals and has been detected in sewage by PCR, but no infectious virus was detected (Wang *et al.*, 2005). The virus appears to survive only a day or two in sewage at room temperature (Wang *et al.*, 2005).

C.   PROTOZOAN PATHOGENS

Cryptosporidium and *Giardia* are the protozoan parasites most often associated with food and waterborne transmission in the United States. They are parasites of the small intestine that cause diarrhea and have environmentally resistant stages called cysts or oocysts. *Cryptosporidium* oocysts and *Giardia* cysts have been detected in products of wastewater treatment, such as anaerobically digested sewage sludge (Chauret *et al.*, 1999; Rimhanen-Finne *et al.*, 2004), and in biosolids (Bean and Brabants, 2001). In one study, the concentration of *Cryptosporidium* oocysts was 2.8 and *Giardia* cysts 12.8 per gram of wet, dewatered, and anaerobically digested biosolids (Chauret *et al.*, 1999). The methods used to detect the cysts and oocysts did not determine viability. The viability of *Cryptosporidium parvum* oocysts, inoculated into aerobic and anaerobic digesters determined by Bowman *et al.* (2000), was investigated. The digesters were maintained at 37°C, 47°C, and 55°C, with 10-day detention times. Oocysts were added to each digester in a single spike or in chambers placed in the digesters for varying periods. Oocysts were inactivated very rapidly in all systems as determined by a dye permeability

assay, >99% inactivated after 10 days at 37°C, 4 days at 47°C, and 2 days at 55°C. Robertson *et al.* (1995) found that 90% of the *Cryptosporidium* oocysts were viable after 18 days of mesophilic digestion and that the oocysts could survive for at least 30 days in sludge-treated soil. However, dye-staining methods were used, which may overestimate viability (Schets *et al.*, 2005). Because oocysts are rapidly inactivated under drying conditions, they would not be expected to survive for very long in low moisture content, such as conditions prevalent during post land application (Robertson *et al.*, 1992).

Overall protozoan cysts appear to present little risk of transmission from land application of biosolids. They are not as heat resistant as the enteric viruses, and are more likely to be inactivated during drying conditions. However, better data, using newer cell-culture methods to assess viability (Slifko *et al.*, 1999), should be conducted on naturally occurring oocysts to confirm this assumption.

Microsporidia are obligate intracellular parasites (e.g., *Encephalitozoon* spp.) that have been associated with gastrointestinal illness in patients with acquired immunodeficiency syndrome (AIDS) and in some healthy individuals. Dowd *et al.* (1998a) confirmed the presence of the human-pathogenic microsporidia *Enterocytozooon bieneusi*, *Encephalitozoon intestinalis*, and *Vittaforma corneae* in water and tertiary sewage effluent. One waterborne outbreak has been described (Cotte *et al.*, 1999). Of over 1200 species described, only 14 have been associated with human infections. At least three of the species that infect humans grow in animal cell culture (Wolk *et al.*, 2000), but no method is available to assess infectivity in environmental samples. The spores of the microsporidia are not unusually resistant to heat (Koudela *et al.,* 1999).

D. Helminths

The US EPA considered the human pathogens *Ascaris lumbricoides*, *Trichuris trichiura*, *Taenia saginata*, *Taenia solium*, *Necator americanus*, and *Hymenolepsis nana* in establishing the pathogen standards of the Part 503 rule. Also included were two animal pathogens *Ascaris suum* (of pigs) and *Toxocara canis* (of dogs). Human infections with *A. lumbricoides*, *T. trichiura*, and *H. nana* are obtained through direct consumption of embryonated eggs. *T. saginata* infections in people are typically acquired from the ingestion of beef, however, the eggs of this organism have been detected in some biosolids (Barbier *et al.*, 1990). The eggs of *Taenia solium* are infectious to pigs, but also are capable of producing larvae that infect people and can cause central nervous system disease (Bale, 2000). Humans who ingest the eggs of *A. suum* of pigs can develop pneumonic, asthmalike signs and can develop a few single

adult worms. People who eat the eggs of *T. canis* can develop visceral or ocular larva migrans, syndromes that occur mainly in children who eat contaminated soil (Overgaauw, 1997; Taylor, 2001).

Worldwide, it has been estimated that 1 billion people are infected with *Ascaris* (Bowman and Fayer, 2005). Although the infection is uncommon in the United States, a study of stools submitted by state diagnostic laboratories in 1987 in the United States indicated an incidence of 8% for *Ascaris lumbrocoides* (Kappus *et al.*, 1994). The concentration and occurrence of *A.* varies widely depending on the incidence in the community from which the sewage is obtained. Studies done before the 503 regulations in the United States found concentrations of viable ova in raw sewage sludge to average 9.6 g per dry weight in the southeastern states and 0.7 g per dry weight in northern states (and that only 48% of the time) (Bowman and Fayer, 2005). In contrast, Jimenez *et al.* (2002) reported concentration of *A.* ova of 66–136 g per dry weight. They also reported significantly greater concentrations of enteric pathogens, probably because of the greater incidence of these infections in Mexico than the United States. In the Czech Republic, Horak (1992) reported $0.024–0.1 \text{ g}^{-1}$ per dry weight. In France, Schwartzbrod and Banas (2003) detected viable nematodes (*Toxocara*, *Trichuris*, *Capillara*, and *Ascaris*) in 69.5% of 194 samples of undigested activated sludge from 20 wastewater treatment plants. The concentration of viable nematode eggs ranged from <0.25 to 7 g per dry weight. In contrast, Thomaz-Soccoi *et al.* (1997) in Brazil, detected 4.85 helminth eggs gram per dry solids in raw sewage sludge.

Ascaris ova are among the most environmentally resistant of intestinal pathogens. In temperate climates, ova have been found to remain viable for up to 7 years in the soil (Smith *et al.*, 1999). The T-90 die-off times for sludge applied to land in Texas, Ohio, and Louisiana was about 3 years (Little *et al.*, 1991). When the sludge was not tilled into the soil, it took only about 2–10 months before 90% of the eggs were inactivated. Mizgajska (1994) showed that eggs applied to the soil tended to stay in the first 10 cm, and no matter what soil type was examined, there were viable eggs present in samples of all soils collected 17 months after application. Mesophilic anaerobic digestion will reduce *Ascaris* egg viability by 30–50% (Bowman and Fayer, 2005), but Class A treatments are needed to cause significant reductions.

T. trichiura (human whipworm) is another helminth having a very environmentally stable egg. Anaerobic and aerobic digestion have no marked effect on its viability, however, Class A treatments should inactivate these parasites (Bowman and Fayer, 2005). Field studies suggest they are capable of remaining viable in soil in temperature climates for at least 1.5–2.5 years (Burden and Hammet, 1979; Burden *et al.*, 1976).

Concerns have been raised about roundworm *Baylisascaris procyonis*. The egg of this worm is similar to that of the related *Ascaris* spp., and the ingestion of the eggs of this parasite can cause severe neurological and ocular disease in humans and has been linked to some fatalities (Sorvillo *et al.*, 2000). However, eggs of *B. procyonis* have not as yet been identified in biosolids.

E. Other Biological Concerns in Biosolids

1. Antibiotic-Resistant Bacteria

Bacteria are prokaryotic organisms with the ability to metabolize and replicate very quickly. They are also very adaptable, genetically. When confronted with an antibiotic, there need only be one bacterial cell with a genetic or mutational change that confers resistance to that antibiotic, which subsequently allows for the proliferation of antibiotic-resistant bacteria (ABRs). Thus, the more that antibiotics are used, the greater the likelihood of antibiotic-resistant strains developing. The greatest concern with antibiotic resistance is the potential for human pathogenic strains to become resistant to overused antibiotics, which subsequently cannot contain the infectious agent. The widespread, sometimes indiscriminant, use of antibiotics has raised the question: "Can antibiotic-resistant genes be transferred from nonpathogenic bacteria to human pathogenic strains?"

Brooks (2004) evaluated the incidence of ABRs in biosolids and a variety of other environmental samples and foodstuffs. Table III and Fig. 2 show that Class B biosolids did not contain unusually high numbers of ABRs, and that in fact, the relative incidence was less than that found in pristine soil. ABR concentrations were also much lower than those found in common foodstuffs. Given that food is consumed to a far greater extent than is physical contact with biosolids, foodstuffs could be a greater threat to human health and welfare than any possible association with biosolids, with respect to ABRs. Finally, note that gene transfer in soil is a relatively infrequent event without selective pressure (Neilson *et al.*, 1994), thereby, further minimizing the risk of antibiotic-resistant gene transfer to human pathogenic bacteria.

2. Endotoxin

Endotoxin, or lipopolysaccharide (LPS) derived from the cell wall of gram-negative bacteria is a highly immunogenic molecule that when introduced directly into the bloodstream has demonstrated the ability to cause a broad range of health effects such as fever, asthma, and shock (hence the

Table III
Comparison of Environmental and Food Samples, Antibiotic-Resistant Percentage
of Total Culturable Heterotrophic Plate Count Bacteria

Sample	Antibiotic resistant (%)			
	Ampicillin[a]	Cephalothin[a]	Ciprofloxacin[a]	Tetracycline[a]
Biosolids	4.3	21.2	1.8	1.9
Composted manure	0.0	0.3	0.0	0.3
Compost	9.7	21.8	3.4	1.2
Fresh manure	0.2	0.7	1.1	0.3
Pristine soil	8.1	10.1	3.1	2.4
Dust	4.9	7.8	8.3	11.2
Ground water	60.3	41.2	22.9	21.0
Raw chicken	47.1	60.3	0.0	0.0
Raw ground beef	16.3	8.7	2.0	3.9
Head lettuce	29.9	35.8	1.5	4.5
Shredded lettuce	14.9	10.5	0.0	0.3
Tomato	0.6	20.6	0.2	0.3
Tap water	6.4	6.8	6.6	7.9

[a]Ampicillin (32 µg ml^{-1}), cephalothin (32 µg ml^{-1}), ciprofloxacin (4 µg ml^{-1}), and tetracycline (16 µg ml^{-1}).
From Brooks (2004).

suffix "toxin") (Bradley, 1979; Michel, 2003; Olenchock, 2001). Lipopoly-saccharide is present ubiquitously throughout the environment, as gram-negative bacteria continuously release LPS during both cell decay and active cell growth. Most surfaces contain some traces of endotoxin due to dust-associated endotoxin, and therefore most human populations come into contact with some endotoxin (Gereda *et al.*, 2001; Sharif *et al.*, 2004). Although endotoxin is present in "everyday" environments, it is primarily of concern as an aerosol, since most human ailments are pulmonary associated.

Exposures to aerosolized endotoxin have been specifically studied regarding occupational exposures from cotton dust, composting plants, and feed houses (Castellan *et al.*, 1987; Clark *et al.*, 1983; Donham *et al.*, 2000; Epstein, 1994; Rylander *et al.*, 1983; Smid *et al.*, 1992). Exposures to levels of endotoxin as little as 0.2 endotoxin unit (EU) m^{-3}, derived from poultry dust, have been found to cause acute pulmonary ailments such as decreases in forced expiratory volume (FEV) (Donham *et al.*, 2000). Chronic effects, such as asthma and chronic bronchitis, have been found to be due to exposures of endotoxin from cotton dust as little as 10 EU m^{-3} on a daily basis (Olenchock, 2001).

Past studies, conducted regarding environmental exposures to endotoxin, have used methods such as membrane trapping of aerosolized endotoxin.

A study compared methods of aerosolized endotoxin collection between traditional membrane trappings and collection via impingement (Duchaine *et al.*, 2001). Results suggest differences between the two methods, and that impingement may result in higher percentage recoveries and greater precision. This study focused on aerosolized endotoxin exposure in occupational settings, specifically swine barns and sawmills. It was shown that swine barns were found to contain mean concentrations of endotoxin ten times greater than that of sawmills, 4385 and 740 EU m^{-3} respectively. Endotoxin concentration ranged from 208 to 17,063 EU m^{-3} for sawmills, and from 2026 to 11,297 EU m^{-3} for swine barns as collected by impingement sampling.

Composting sites have also been studied with respect to endotoxin exposures; although most sites have been shown to contain concentrations of aerosolized endotoxin greater than that of background levels, these levels were thought to be within safe limits, <1000 EU m^{-3} (Rylander *et al.*, 1983). It was suggested by the authors that no more than a maximum air concentration of 1000 EU m^{-3} should be considered safe until additional studies have been conducted. A study conducted by Clark *et al.* (1983) determined aerosolized endotoxin concentrations from a composting plant to be between 10 and 400 EU m^{-3}. It is important to note that despite the presence of endotoxin within these sites, there was no evidence of residential impact, since beyond the composting site boundaries levels regressed to background concentrations.
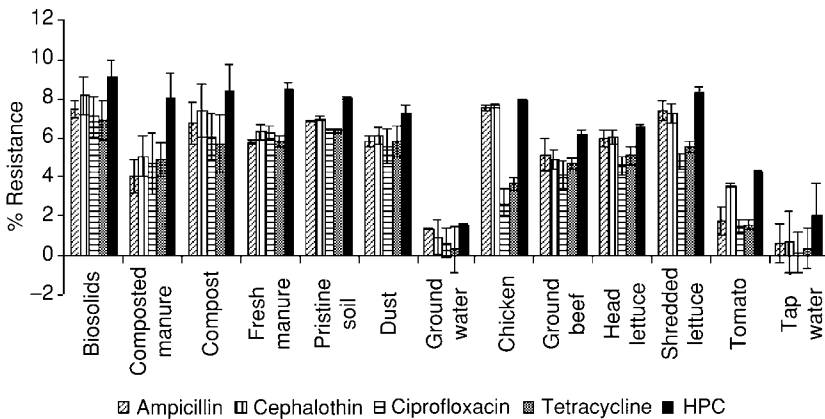
Figure 2 Heterotrophic plate count bacteria (HPC) and antibiotic-resistant bacteria from common consumables and environmental samples. Resistant Antibiotic Concentrations: ampicillin (32 μg ml^{-1}), cephalothin (32 μg ml^{-1}), ciprofloxacin (4 μg ml^{-1}), and tetracycline (16 μg ml^{-1}). From Brooks (2004).

Table IV
Aerosolized Endotoxin Concentrations Detected Downwind of Biosolids Operations,
a Wastewater Treatment Plant Aeration Basin, and a Tractor Operation

Sample type	Number of samples collected	Distance from site (m)	Avg[a]	Median (EU m^{-3a})	Minimum (EU m^{-3a})	Maximum (EU m^{-3a})
				Aerosolized endotoxin		
Background	12	NA[a]	2.6	2.49	2.33	3.84
Biosolids operations						
Loading	39	2–50	343.7	91.5	5.6	1807.6
Slinging	24	10–200	33.5	6.3	4.9	14.29
Biosolids pile	6	2	103	85.4	48.9	207.1
Total operation	33	10–200	133.9	55.6	5.6	623.6
Wastewater treatment plant						
Aeration basin	6	2	627.3	639	294.4	891.1
Nonbiosolids field						
Tractor	6	2	469.8	490.9	284.4	659.1

[a]Avg—average; EU m^{-3}—endotoxin units per cubic meter; NA—not applicable.
Source: Brooks, J. P. (2004). "Biological aerosols generated from the land application of biosolids: Microbial Risk, Assessment." Ph.D. Dissertation, The University of Arizona, Tucson, AZ.

Endotoxin concentrations in a variety of environmental samples were investigated by Brooks *et al.* (2006) who showed that the endotoxin levels in Class B biosolids were similar in magnitude to that of other wastes, including animal manures and compost. Since the relevance of this to human health is via inhalation, the potential for aerosolization of endotoxin during land application has also caused concern.

In soils, bacterial concentrations routinely exceed 10^8 g^{-1}, with a majority of bacteria being gram-negative. Soil particles containing sorbed microbes can be aerosolized and hence act as a source of endotoxin. Farming operations, such as operating a tractor across a field, have been shown to result in endotoxin levels of 469 EU m^{-3} (Brooks *et al.*, 2006). These values are comparable to those found during land application of biosolids operations (Table IV). Therefore, farming operations can result in endotoxin aerosolization, regardless of whether biosolids are involved.

3. Prions

Concern about prions has arisen with the advent of prion animal diseases, such as bovine spongiform encephalopathy (BSE), in the United Kingdom and other parts of Europe. The BSE prions concentrate in an animal's brain and spinal cord, but they have been detected only in sheep blood at low

concentrations. Animal manure would have no or low concentrations of BSE prions except possibly for wastes from slaughterhouses (Ward *et al.*, 1984). However, the presence of prions in such wastes is uncertain (EPA, 2001). Prions are generally transmitted from animal to animal (cow to cow, sheep to sheep). The risk of prion transmission to biosolids from animals is low but can increase with the presence of small amounts of neural tissues or placenta coming from slaughter houses. At present, there has been little evidence of prion-contaminated manures in the United States.

Prions are very difficult to inactivate and require rigorous treatment (Godfree, 2001). The higher the solids content of the waste, the more rigorous the treatment required (EPA, 2001). Prions are resistant to high temperatures; scrapie prions are inactivated at temperatures of $100\,^{\circ}C$ or above. At $121\,^{\circ}C$, only 0.01% of the prions were resistant to thermal inactivation (Rohwer, 1984). Prions have been reported to survive boiling and autoclaving (EPA, 2001). Prion survival at increased temperatures, coupled with chemical or biological treatment associated with biosolids processing has not been studied. In addition, no data are available to directly assess prion survival through sewage-sludge treatment processes.

Gale and Stanfield (2001) conducted a risk analysis on the potential for the transmission of BSE to cattle and via vegetables from the land application of sewage sludge after treatment. The source of the BSE was assumed to be abattoirs.

VI. PATHOGEN TRANSPORT AND SURVIVAL IN SOIL, WATER, AND AIR

Pathogens that are present in biosolids which are land applied can result in human exposure via soil, water or air. Therefore a discussion of pathogen survival and transport in these media are warranted.

A. Exposure via Soil and Groundwater

Soil affects human exposure to pathogens in two ways. First, survival times of pathogens in soil amended with biosolids affect the potential for human contact subsequent to land application. This aspect was the driving force behind implementation of site restrictions following land application. Soil also directly affects the potential for transport of pathogens to underground aquifers.

Pathogen survival in and transport through soil are considered together in this section. Environmental factors that affect survival of pathogens are

summarized in Table V. Human pathogens that are routinely found in domestic sewage sludge include viruses, bacteria, protozoan parasites, and helminths. Of these pathogens, viruses are the smallest and least complex; they generally have a short survival; and the greatest potential for transport in soil. Survival of virus has been shown to be temperature-dependent and decreased as temperature increased (Straub *et al.*, 1993a). Soil type affected virus survival, longer survival occurring on clay loam biosolids-amended soils compared to sandy loam biosolids-amended soils (Straub *et al.*, 1993b). Rapid loss of soil moisture also limited virus survival. When conventional cultural methods are used, virus survival ranges from 3 days to greater than 10 days, depending on soil type, temperature, and moisture (Straub *et al.*, 1992, 1993a). When molecular PCR-based methods are used, enteroviruses can be detected in soil 3 months after land application (Straub *et al.*, 1995). However, PCR by itself only detects viral nucleic acid and does not indicate that viable viruses were actually present.

Like virus survival, bacteria survival in soil is affected by temperature, pH, and moisture (Gerba *et al.*, 1975). Soil nutrient availability also plays a role in bacteria survival. Lower temperatures usually increase survival, as do a neutral soil pH and soil at field capacity with respect to moisture. Of the pathogenic bacteria, *E. coli* can survive for long periods in biosolids-amended soil. *Salmonella* was shown to survive for 2–3 weeks in biosolid-amended soil (Zaleski *et al.*, 2005a). In contrast, *Shigella* has a shorter survival time than either *Salmonella* or *E. coli* (Feachem *et al.*, 1983). Studies on indicator organisms have shown that total and fecal coliforms as well as fecal streptococci can all survive for weeks to several months depending on soil moisture and temperature conditions (Pepper *et al.*, 1993).

Regrowth is also important when evaluating the survival of pathogenic and indicator bacteria in soil and biosolids compost (Zaleski *et al.*, 2005b). *Salmonella*, *E. coli*, and fecal coliforms are all capable of regrowth.

Table V
Environmental Factors Affecting the Survival of Pathogenic Microbes

Parameter	Survival time		
	Virus	Bacteria	Protozoa
Temperature increasing	−	−	−
Soil moisture decreasing	−	−	−
Rate of desiccation increasing	−	−	−
Clay content increasing	+	+	Not known
pH range of 6–8	+	+	+

−, decreasing survival time; +, increasing survival time.
Source: Adapted from NRC, 2002.

Following land application of biosolids or composting of biosolids with soil, pathogen concentrations decrease below the detection limit but can increase after rainfall. Regrowth is generally limited to Class A biosolids where biological competition is less than in Class B biosolids (Zaleski *et al.*, 2005b). Zaleski *et al.* (2005a) demonstrated that *Salmonella* would not regrow in soil amended with biosolids.

The protozoan parasites often associated with biosolids include *Giardia* and *Cryptosporidium* spp. However, little research has been conducted on the survival of these parasites in biosolids-amended soil. Helminths are perhaps the most persistent of enteric pathogens. *Ascaris* eggs can survive for several years in soils at low temperatures (Straub *et al.*, 1993b).

The transport of microorganisms through soils or vadose zone materials is affected by a complex array of abiotic and biotic factors, including adhesion processes, filtration effects, physiological state of the cells, soil characteristics, water flow rates, predation, and intrinsic mobility of the cells (Newby *et al.*, 2000). For viruses, the potential for transport is potentially significant, although viruses can adsorb to soil colloidal particles and to the biosolids themselves, thus limiting transport (Schijven and Rietveld, 1996). Virus sorption is controlled by the soil pH. Most viruses are negatively charged (isoelectric point 3–6) so that at a neutral soil pH, soil sorption is reduced, whereas at more acidic soil pH values the viruses are positively charged, increasing sorption (Dowd *et al.*, 1998b). Chetochine *et al.* (2006) showed that viruses are embedded and/or adsorbed to biosolids. Studies with phage showed that this sorption appeared to influence the potential for release and subsequent transport of the virus through soil under saturated conditions. Overall less than 8% of the indigenous coliphage initially present in the biosolids were leached out of the biosolids soil matrix. Human enteric viruses appear to be tightly bound to biosolids because bacteria that grow in the activated sludge process produce proteins, which bind the virus to the biosolid matrix (Sano *et al.*, 2004). Thus, groundwater contamination from land-applied biosolids does not appear to be likely.

B. Exposure via Air

Exposure to pathogens or other biological entities, such as endotoxin, can occur via transport through the air as bioaerosols. Bioaerosols consist of microorganisms or other biological particles such as endotoxin or peptidoglycans that become airborne with the potential to be transported over significant lateral distances. If the microbes transported are pathogenic then exposure to them potentially becomes a human health issue. The potential for aerosolization of pathogens from land application of biosolids has become an issue that has been debated nationally. To date, few studies

on land application of biosolids have been conducted, but several studies have evaluated aerosols from wastewater treatment plants, land application of wastewater, animal manures, and composting operations. Overall, the potential for adverse health effects from pathogens in aerosols depends on their fate and transport. The fate and inactivation of aerosolized microbes is affected by numerous environmental factors and methods of aerosol generation, while transport, or the lateral distance aerosols are carried from source to endpoint, is affected by factors such as wind direction and velocity.

A limited number of studies have been conducted on the generation of bioaerosols from biosolids land application. Notably, Sorber *et al.* (1984) concluded that little or no risk was associated with the land application of liquid biosolids, based on the lack of viral presence in large volumes of sampled air. Other studies have focused on large piles of biosolids, unloaded by trucks on site and subsequently loaded with front-end loaders into biosolids spreaders or hoppers (Dowd *et al.*, 2000; Pillai *et al.*, 1996). Loading events proved to be sources of increased concentrations of fecal microbial indicators such as H_2S producing bacteria and *Clostridium* spp. No risk analyses were conducted in the previous study, although the investigators concluded that the microbial indicator concentrations were below levels that could be construed as a risk to public health. The latter study conducted microbial risk analyses based on the use of complex transport models first proposed for the transport of chemical aerosols (Pasquill, 1961). Through the use of these models, aerosol concentrations could effectively be predicted at downwind distances from both point (biosolids pile) and area sources (a biosolids applied field) (Dowd *et al.*, 2000). Conservative occupational risk analysis was conducted and risk calculations ranged from a 3% chance of infection to a 100% chance of infection based on infection from aerosolized Coxsackie virus.

In the Dowd *et al.* (2000) study, a ''worse case'' scenario during land application of biosolids predicted a risk of infection of 1.00 (100%). However an incorrect infectivity constant was used in this calculation. Using the correct and more realistic values of phage—human virus ratios, the predicted risk is 5 orders of magnitude less than 1.00 (Brooks *et al.*, 2004).

In 2002, the National Research Council identified the potential for human exposure to pathogens via bioaerosols generated during land application, to be priority research item. In response to this, a major national study in the United States was initiated by the University of Arizona. The goals of the study were to evaluate the ''community'' and ''occupational'' risk of infection from bioaerosols. This study evaluated the incidence of bioaerosols across the United States with differing climatic conditions and various methods of biosolid application, and more than 1000 aerosol samples were collected and analyzed for bacterial and viral pathogens. The risk assessment

from this study is presented in Section VII. The conclusions of this study were that the overall community risk of infection from bioaerosols during land application was relatively negligible. Occupational risks during land application were found to be greater than community risks, due to higher exposure, but calculated risks were still low (Brooks *et al.*, 2004a,b). Bioaerosol emission rates and plume characteristics during land application were also determined (Tanner *et al.*, 2005).

VII. RISK-BASED EVALUATION OF THE POTENTIAL HAZARDS POSED BY PATHOGENS IN BIOSOLIDS

The process of quantitative microbial risk assessment is an approach designed to estimate the risk of infection and adverse outcomes from the environmental exposure of pathogens (Rose and Gerba, 1991). The risk assessment approach can be used to answer ("What if") questions such as how many people will be infected if untreated sewage sludge is used on vegetables to be eaten raw. It has also been used to assess risks from emerging pathogens and as guidance for the establishment of drinking water treatment standards in the United States (Regli *et al.*, 1991). It has been used to assess microbial risks from exposure to land-applied biosolids (Gale, 2005; Gerba *et al.*, 2002; Westrell *et al.*, 2004).

A. On-Site Exposure from Land-Applied Biosolids

Because Class B biosolids contain human pathogenic microorganisms, site restrictions on land applied with Class B biosolids were implemented via the 503 regulations (EPA, 1993). These site restrictions regulate the minimum duration between application and the harvesting of crops, grazing access, and public access (Table VI). Therefore, in theory, sufficient time elapses between land application and subsequent human contact that all pathogenic microbes have been inactivated via natural attenuation. However, because public access may occur prior to the minimum time requirement, then a risk analysis is warranted. Box 7 illustrates the risk of infection from ingesting biosolids, while Box 8 illustrates the risk of infection from ingesting land-applied biosolids.

In both examples, the risk of infection from rotavirus is relatively low. For comparison, note that the EPA guidelines for safe drinking water with respect to pathogens are an annual risk of no greater than 10^{-4} (Regli *et al.*, 1991).

<div align="center">

Table VI
Minimum Duration Between Application and Harvest/Grazing/Access for Class B
Biosolids Applied to the Land

</div>

Criteria	Surface	Incorporation	Injection
Food crops whose harvested part may touch the soil/biosolids mixture (beans, melons, squash, and so on)	14 months	14 months	14 months
Food crops whose harvested parts grow in 20/38 months[a]	20/38 months[a]	38 months	38 months
Food, feed, and fiber crops (field corn, hay, sweet corn, and so on)	30 days	30 days	30 days
Grazing of animals	30 days	30 days	30 days
Public access restriction			
High potential[b]	1 year	1 year	1 year
Low potential	30 days	30 days	30 days

[a]The 20-months duration between application and harvesting applies when the biosolids that are surface applied stays on the surface for 4 months or longer prior to incorporation into the soil. The 38 month duration is in effect when the biosolids remain on the surface for less than 4 months prior to incorporation.
[b]This includes application to turf farms which place turf on land with a high potential for public exposure.
Source: Adapted from 40 CFR, Part 503.

Box 7
Probability of Infection from Ingesting Biosolids

Assumptions:

A. A child jumps into a pile of Class B biosolids and plays for 8 h.
B. Anaerobically digested biosolids containing 0.12 rotavirus per 4 g biosolids.
C. The child ingests 50 mg of biosolids.

Risk of infection $= 8.6 \times 10^{-4}$

B. ON-SITE EXPOSURE TO WORKERS VIA BIOAEROSOLS GENERATED DURING LAND APPLICATION OF BIOSOLIDS

The potential for on-site exposure to aerosolized pathogens is maximized for biosolid workers involved in loading and unloading biosolids, and also during actual land application. Because of their occupation, such workers have enhanced exposure and hence a greater risk of infection via aerosols.

Box 8
Probability of Infection from Ingesting Land-Applied Biosolids

Assumptions:

A. A child plays for 8 h in a field immediately after Class B biosolids have been applied.
B. The concentration of biosolids is diluted 100-fold by the soil.
C. The anaerobically digested biosolids contains 0.12 rotavirus per 4 g biosolids.
D. The child ingests 50 mg of biosolid amended soil.

Risk of infection from rotavirus $= 1.8 \times 10^{-6}$

Tanner (2004) evaluated the occupational risk to biosolid workers based on the University of Arizona study on bioaerosols. As part of this study, bioaerosol emission rates and plume characteristics generated during the land application of liquid Class B biosolids were also investigated (Tanner *et al.*, 2005). The number of viral pathogens generated on site during land application of biosolids was determined to be 3.3×10^{-3} viral pathogens per cubic meter of air (downwind of the site). Likewise, the number of bacterial pathogens was determined to be 1×10^{-3} m^{-3} of air. Risk assessments for the probability of infection from nontyphi *Salmonella* and Coxsackie virus A21 were conducted (Tanner, 2004). The annual occupational risk of infection from Coxsackie virus A21 was found to be 2×10^{-2}. In contrast, the annual risk of infection from non-typhi *Salmonella* was 1.3×10^{-4}).

For non-typhi *Salmonella*, no dose response information for inhalation was available since human transmission by inhalation of aerosols has not been demonstrated, therefore, risks were calculated on the assumption that 10% of bacterial aerosols would impact in the throat and be ingested following inhalation. The Beta Poisson Dose Response Model provides the best estimate of the probability of infection from ingestion of pathogenic enteric bacteria and assumes that a single surviving bacterium is sufficient to cause infection (Holcomb *et al.*, 1999; Teunis *et al.*, 1999).

C. OFF-SITE EXPOSURE OF BIOAEROSOLS TO RESIDENTS IN COMMUNITIES CLOSE TO LAND APPLICATION SITES

The risk of infection of residents living close to land application sites via bioaerosols was identified as an item of concern in the NRC report on land application (NRC, 2002). Community risks were evaluated in the University of Arizona study (Brooks *et al.*, 2004, 2005a,b).

<div style="text-align:center">

Table VII
Annual Community Risk of Infection from Coxsackie Virus A21

</div>

Annual risk					
		Number of virus			
10 virus g^{-1} biosolid		1 virus g^{-1} biosolid		0.1 virus g^{-1} biosolid	
		Exposure time			
1 h	8 h	1 h	8 h	1 h	8 h
4.7×10^{-5}	3.8×10^{-4}	4.7×10^{-6}	3.8×10^{-5}	4.7×10^{-7}	3.8×10^{-6}

For community risk, fate and transport of pathogens is a factor since residents live off-site, allowing for natural attenuation of pathogens due to environmental factors such as dessication and ultraviolet light. The annual community risk of infection from Coxsackie virus A21 was determined using the one-hit exponential model (Brooks *et al.*, 2005a,b) and is shown in Table VII. Based on these data, community risks from bioaerosols generated during land application of biosolids are very low.

VIII.   PUBLIC PERCEPTIONS OF LAND APPLICATION OF BIOSOLIDS WITH RESPECT TO PATHOGENS

Public perception of land application of biosolids with respect to pathogens is in part a function of land availability and population density. For example, in the desert southwest, agricultural areas are often located far from urban centers, so that there are fewer surrounding residents, who come into contact with the process. In contrast, in the northeast, the potential impact of land application is much greater, because of less land and larger populations (NRC, 2002). In the vast majority of cases, it is odor that is the initial cause of complaints.

In the early 2000s, when the Internet became a viable method of quick communication, concerns were aggressively raised by environmental activists with respect to the potential for human infections from bioaerosols. In particular, the potential for concern over *Staphylococcus aureus* infections became a preeminent issue for those opposed to land application of biosolids. Events came to a head in the summer of 2002, when a National Academy of Science report on biosolid land application was released (NRC, 2002). The most noted quote from the report was—''there is no documented scientific evidence that the Part 503 rule has failed to protect

human health. However, additional scientific work is needed to reduce persistent uncertainty about the potential for adverse human health effects from exposure to biosolids.''

The overarching recommendations of the report were as follows:

- Use improved risk-assessment methods to better establish standards for chemicals and pathogens.
- Conduct a new national survey of chemicals and pathogens in sewage sludge.
- Establish a framework for an approach to implement human health investigations.
- Increase the resources devoted to EPA's biosolids program.

These recommendations have yet to be implemented.

Vocal opposition to land application of biosolids has been in the State of California, where several counties now allow only land application of Class A biosolids. In addition, several lawsuits have been filed that claimed that deaths were due to land application of biosolids. To date, no ''cause and effect'' has ever been shown due to land application of biosolids. Fears of *S. aureus* infections were allayed due to evidence that *S. aureus* is absent in biosolids (Rusin *et al.*, 2003). In addition, new research has shown that community risk for bioaerosol infections is negligible (Brooks *et al.*, 2005a,b; Tanner *et al.*, 2005).

Overall, about 60% of all biosolids produced are land applied in both the United States and United Kingdom. The vast majority of this is Class B biosolids. Since other methods of disposal, such as incineration or landfilling have serious drawbacks, then reuse via land application is likely to continue as the most viable option. That not withstanding, there will continue to be a need for state-of-the-art research, as new issues emerge.

IX. FUTURE RESEARCH NEEDS

There will always be a need for an understanding of the occurrence of pathogens in biosolids and the effectiveness of treatment processes. New pathogens will continue to emerge and methods for their detection in the environment will be developed. Newer and better treatment processes will be developed, which require assessment for pathogen removal. Data on the concentration of pathogens in biosolids is critical to conducting quantitative estimates of risks from the land application of biosolids.

Most of our understanding on virus removal by biosolid-treatment processes and environmental fate rests largely upon studies conducted with enteroviruses. Since many of the biosolid-treatment processes depend on temperature and high pH, we need to assess the fate of viruses that demonstrate the most resistance to these processes, that is, hepatitis A

virus and adenoviruses. The potential for regrowth of *E. coli* 0157:H7 in compost or after other treatment processes needs assessment. The fate of *Cryptosporidium* oocysts during treatment and after land application, using newer viability methods needs to be conducted to ensure that risks are minimal under a variety of environmental conditions after land application.

Because many of the limits for use of agricultural land after land application of Class B are determined by the survival of *Ascaris* ova, a proper risk assessment needs to be conducted to determine more quantitatively the risks involved. This will require data on current levels of viable ova in biosolids and their survival under different environmental conditions. Finally, the incidence of prions in biosolids needs to be determined.

REFERENCES

Al-Ghazali, M. R., and Al-Azawi, S. K. (1990). *Listeria monocytogenes* contamination of crops grown on soil treated with sewage sludge cake. *J. Appl. Bacteriol.* **69**, 642–647.

Ansari, S. A., Farrah, S. R., and Chaudhry, G. R. (1992). Presence of human immunodeficiency virus nucleic acids in wastewater and their detection by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**, 3984–3990.

Arvanitidou, M., Mamassi, P., and Vayona, A. (2004). Epidemiological evidence for vaccinating wastewater treatment plant workers against hepatitis A and hepatitis B virus. *Eur. J. Epidemiol.* **19**, 359–362.

Bale, J. F., Jr. (2000). Cysticercosis. *Curr. Treat. Options Neurol.* **2**, 355–360.

Barbier, D., Perrine, D., Duhamel, C., Doublet, R., and Georges, P. (1990). Parasitic hazard with sewage sludge applied to land. *Appl. Environ. Microbiol.* **56**, 1420–1422.

Bean, C. L., and Brabants, J. J. (2001). A survey of wastewater solids to assess the prevalence of *Cryptosporidium*, *Giardia* species, and *Ascaris lumbricoides*: An evaluation of risks associated with land application of biosolids. Poster presented at the 101st General Meeting of the American Society of Microbiology, May 23, 2001. Orlando, FL.

Bhaduri, S., Wesley, I. V., and Bush, E. J. (2005). Prevalence of pathogenic *Yersina enterocolitica* strains in pigs in the United States. *Appl. Environ. Microbiol.* **71**, 7117–7121.

Biagini, P. (2004). Human circoviruses. *Vet. Microbiol.* **98**, 95–101.

Bofill-Mas, S., Clemente-Casares, P., Major, E. O., Curfman, B., and Girones, R. (2003). Analysis of the excreted JC virus strains and their potential oral transmission. *J. Neurovirol.* **9**, 498–507.

Bowman, D. D., and Fayer, R. (2005). Concerns related to protozoan and helminth parasites in biosolids and animal wastes. *In* "Infectious Disease Agents Ion Sewage Sludge and Manure" (J. E. Smith, P. D. Millner, W. Jakubowski, N. Goldstein, and R. Rynak, Eds.), pp. 127–162. The JG Press, Emmaus, PA.

Bowman, D. D., Reimers, R. S., Little, M. D., Jenkins, M. B., Bankston, W. S., and Atique, M. M. (2000). Assessment and comparison of ascaris egg and *Cryptosporidium* oocyst inactivation with respect to biosolids processing. 14th Annual Residuals and Biosolids Management Conference, February/March 2000. Specialty conf. paper. Water Environment Federation, Alexandria, VA.

Bradley, S. G. (1979). Cellular and molecular mechanisms of action of bacterial endotoxins. *Ann. Rev. Microbiol.* **33**, 67–94.

Brooks, J. P. (2004). Biological aerosols generated from the land application of biosolids: Microbial risk assessment. (Ph.D. Dissertation). University of Arizona, Tucson, AZ.

Brooks, J. P., Gerba, C. P., and Pepper, I. L. (2004). Biological aerosol emission, fate, and transport from municipal and animal wastes. *J. Residuals Sci. Technol.* **1**, 15–28.

Brooks, J. P., Tanner, B. D., Gerba, C. P., Haas, C. N., and Pepper, I. L. (2005a). Estimation of bioaerosol risk of infection to residents adjacent to a land-applied biosolids site using an empirically derived transport model. *J. Appl. Microbiol.* **98**, 397–405.

Brooks, J. P., Tanner, B. D., Josephson, K. L., Gerba, C. P., Haas, C. N., and Pepper, I. L. (2005b). A national study on the residential impact of biological aerosols from the land application of biosolids. *J. Appl. Microbiol.* **99**, 310–322.

Brooks, J. P., Tanner, B. D., Gerba, C. P., and Pepper, I. L. (2006). The measurement of aerosolized endotoxin from land application of Class B biosolids in Southeast Arizona. *Can. J. Microbiol.* **52**, 150–156.

Brown, L. M. (2000). *Helicobacter pylori*: Epidemiology and routes of transmission. *Epidemiol. Rev.* **22**, 283–297.

Buras, N. (1974). Recovery of viruses from waste-water and effluent by the direct inoculation method. *Water Res.* **8**, 19–22.

Burden, D. J., and Hammet, N. C. (1979). Development and survival of *Trichuris suis* ova on pasture plots in the south of England. *Res. Vet. Sci.* **29**, 66–70.

Burden, D. J., Whitehead, A., Green, E. A., McFadzean, J. A., and Beer, R. J. S. (1976). The treatment of soil infested with human whipworm *Trichuris trichiura*. *J. Hyg.* **77**, 377–382.

Castellan, R. M., Olenchock, S. A., Kinsley, K. B., and Hankinson, J. L. (1987). Inhaled endotoxin and decreased spirometric values: An exposure-response relation for cotton dust. *New Engl. J. Med.* **317**, 605–610.

Chapron, C. D., Ballester, N. A., and Margolin, A. B. (2000). The detection of astrovirus in sludge biosolids using an integrated cell culture nested PCR technique. *J. Appl. Microbiol.* **89**, 11–15.

Chauret, C., Springthorpe, S., and Sattar, S. (1999). Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. *Can. J. Microbiol.* **45**, 257–262.

Chetochine, A. S., Brusseau, M. L., Gerba, C. P., and Pepper, I. L. (2006). Leaching of phage from Class B biosolids and potential transport through soil. *Appl. Environ. Microbiol.* **72**, 665–671.

Clark, C. S., Rylander, R., and Larsson, L. (1983). Levels of gram-negative bacterial, *Aspergillus fumigatus*, dust and endotoxin at compost plants. *Appl. Environ. Microbiol.* **45**, 1501–1505.

Cotte, L., Rabodonirinia, M., Chapuis, F., Bailly, F., Bissuel, F., Raynal, C., Gelas, P., Persar, F., Piens, M. A., and Trepo, C. (1999). Waterborne outbreak of intestinal microsporifiosis in persons with and without human immunodeficiency virus infection. *J. Infect. Dis.* **180**, 2003–2008.

Croci, L., Ciccozzi, M., DeMedici, D., DiPasquale, S., Fiore, A., Mele, A., and Toti, L. (1999). Inactivation of hepatitis A virus in heat-treated mussels. *J. Appl. Microbiol.* **87**, 884–888.

Dawson, S., McArdle, F., Bennett, D., Carter, S. D., Bennett, M., Ryvar, R., and Gaskell, R. M. (1993). Investigation of vaccine reactions and breakdowns after feline calicivirus vaccination. *Vet. Rec.* **132**, 346–350.

DeLuca, G., Zanetti, F., Fateh-Moghadm, P., and Stampi, S. (1998). Occurrence of *Listeria monocytogenes* in sewage sludge. *Zentralbl. Hyg. Umweeltmed.* **201**, 269–277.

Deneen, V. C., Hunt, J. M., Paule, C. R., James, R. I., Johnson, R. G., Raymond, M. J., and Hedberg, C. W. (2000). The impact of foodborne calicivirus disease: The Minnesota experience. *J. Infect. Dis.* **181**(Suppl. 2), S281–S283.

Donham, K. J., Dumro, D., Rynolds, S. J., and Merchant, J. A. (2000). Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in

poultry workers: Recommendations for exposure limits. *J. Occup. Environ. Med.* **42**, 260–269.

Dowd, S. E., Gerba, C. P., and Pepper, I. L. (1998a). Confirmation of the human-pathogenic Microsporidia *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Vittaforma corneae* in water. *Appl. Environ. Microbiol.* **64**, 3332–3335.

Dowd, S. E., Pillai, S. D., Wang, S., and Corapcioglu, M. Y. (1998b). Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. *Appl. Environ. Microbiol.* **64**, 405–410.

Dowd, S. E., Gerba, C. P., Pepper, I. L., and Pillai, S. D. (2000). Bioaerosol transport modeling and risk assessment in relation to biosolid placement. *J. Environ. Qual.* **29**, 343–348.

Duchaine, C., Thorne, P. S., Merizux, A., Grimard, Y., Whitten, P., and Cormier, Y. (2001). Comparison of endotoxin exposure assessment by bioaerosol impinger and filter-sampling methods. *Appl. Environ. Microbiol.* **67**, 2775–2780.

Enriquez, C. E., Hurst, C. J., and Gerba, C. P. (1995). Survival of the enteric adenoviruses 40 and 41 in tap, sea, and wastewater. *Wat. Res.* **29**, 2548–2553.

EPA (US Environmental Protection Agency). (1993). The standards for the use or disposal of sewage sludge. Final 40 CFR Part 503 Rules. EPA 822/Z–93/001.

EPA (US Environmental Protection Agency). (1994). A plain English guide to the EPA Part 503 Biosolids Rule. EPA 832/R–93/003.

EPA (US Environmental Protection Agency). (1995). A guide to the biosolids risk assessments for the EPA Part 503 Rule. EPA 832–B–93–005. Available on line: http://www.epa.gov/owm/bio/503rule/index.htm.

EPA (US Environmental Protection Agency). (2000). A guide to field storage of biosolids and the organic by–products used in agriculture and for soil resource managementUnited States Environmental Protection Agency, Office of Wastewater Management, EPA/832–B–00–007, Washington, DC.

EPA (US Environmental Protection Agency). (2001). Workshop on emerging infectious disease agents associated with animal manures, biosolids and other similar by-products, Cincinnati, OH, June 4–6, 2001, National Risk Management Research Laboratory, US Environmental Protection Agency, Cincinnati, OH.

Epstein, E. (1994). Composting and bioaerosols. *Biocycle* **35**, 51–58.

Feachem, R. G., Brandley, D. J., and Garelick, H. (1983). Sanitation and disease: Health aspects of excreta and waste management. Wiley, New York.

Gale, P. (2005). Land application of treated sewage sludge: Quantifying pathogen risks from consumption of crops. *J. Appl. Microbiol.* **98**, 380–396.

Gale, P., and Stanfield, G. (2001). Towards a quantitative risk assessment for BSE in sewage sludge. *J. Appl. Microbiol.* **91**, 563–569.

Gerba, C. P., Wallis, C., and Melnick, J. L. (1975). Fate of wastewater bacteria and viruses in soil. *Proc. ASCE J. Irrig. Drain. Div.* **101**, 157–174.

Gerba, C. P., Rose, J. B., and Haas, C. N. (1996). Sensitive populations: Who is at the greatest risk? *Int. J. Food Microbiol.* **30**, 113–123.

Gerba, C. P., Pepper, I. L., and Whitehead, L. F. (2002). A risk assessment of emerging pathogens of concern in the land application of biosolids. Sludge management, regulation, utilization and disposal. *Water Sci. Technol.* **46**, 225–230.

Gereda, J. E., Klinnert, M. D., Price, M. R., Leung, D. Y. M., and Liu, A. H. (2001). Metropolitan home living conditions associated with indoor endotoxin levels. *J. Allergy Clin. Immun.* **107**, 790–796.

Godfree, A. (2001). Control of pathogens. Workshop on emerging infectious disease agents associated with animal manures, biosolids and other similar by-products, Cincinnati, Ohio, June 4–6, 2001, National Risk Management Research Laboratory, US Environmental Protection Agency, Cincinnati, OH.

Graff, J., Ticehurst, J., and Flehing, B. (1993). Detection of hepatitis-A in sewage-sludge by antigen capture polymerase chain-reaction. *Appl. Environ. Microbiol.* **59**, 3165–3170.

Green, K. H., Ando, T., Balayan, M. S., Berke, T., Clarke, I. N., Estes, M. K., Matson, D. O., Nakata, S., Neill, J. D., Studdert, M. J., and Thiel, H.-J. (2000). Taxonomy of the caliciviruses. *J. Infect. Dis.* **181**(Suppl. 2), S322–S330.

Gregersen, P., Grunnet, K., Uldum, S. A., Andersen, B. H., and Madsen, H. (1999). Pontiac fever at a sewage treatment plant in the food industry. *Scand. J. Work Environ. Health* **26**, 291–295.

Hejkal, T. W., Smith, E. M., and Gerba, C. P. (1984). Seasonal occurrence of rotavirus in sewage. *Appl. Environ. Microbiol.* **47**, 588–590.

Hinesly, T. D., Jones, R. L., and Ziegler, E. L. (1972). Effects on corn by application of heated anaerobically digested sludge. *Compost Sci.* **13**, 26–30.

Holcomb, D. L., Smith, M. A., Ware, G. O., Hung, Y., Brackett, R. E., and Doyle, M. P. (1999). Comparison of six dose-response models for use with food-borne pathogens. *Risk Anal.* **19**, 1091–1100.

Horak, P. (1992). Helminth eggs in the sludge from three sewage-treatment plants in Czechoslovakia. *Folia Parasit.* **39**, 153–157.

Horan, N. J., Fletcher, L., Betmal, S. M., Wilks, S. A., and Keevil, C. W. (2004). Die-off of enteric bacterial pathogens during mesophilic anaerobic digestion. *Water Res.* **38**, 1113–1120.

Huang, P. W., Laborde, D., Land, V. R., Matson, D. O., Smith, A. W., and Jiang, X. (2000). Concentration and detection of caliciviruses in water samples by reverse transcription-PCR. *Appl. Environ. Microbiol.* **66**, 4383–4388.

Hurst, C. J., McClellan, K. A., and Benton, W. H. (1988). Comparison of cytopathogenicity, immunofluorescence and *in situ* DNA hybridization as methods for the detection of adenoviruses. *Water Res.* **22**, 1547–1552.

Jimenez, B., Maya, C., Sanchez, E., Romero, A., Lira, L., and Barrios, J. A. (2002). Comparison of the quantity and quality of the microbiological content of sludge in countries with high and low content of pathogens. *Water Sci. Technol.* **46**, 17–24.

Jones, K., Betaieb, M., and Telford, D. R. (1990). Seasonal variation of thermophilic campylobacters in sewage sludge. *J. Appl. Bacteriol.* **69**, 185–189.

Kappus, K. D., Lundgren, R. G., Juranek, D. D., Roberts, J. M., and Spencer, H. C. (1994). Intestinal parasitism the United States: Update on a continuing problem. *Am. J. Trop. Hyg.* **50**, 705–713.

Kasorndorkbua, C., Opriessing, T., Huang, E. F., Guenette, D. K., Thomas, P. J., Meng, X. J., and Halbur, P. G. (2005). Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking. *Appl. Environ. Microbiol.* **71**, 7831–7837.

Koudela, B., Kucerova, S., and Hudcovic, T. (1999). Effect of low and high temperatures on infectivity of *Encephalitozoon cuniculi* spores suspended in water. *Folia Parasit.* **46**, 171–174.

Kukkula, M., Arstila, P., Klossner, M. L., Mznnula, L., Bonsdorff, C. H., and Jaatinen, P. (1997). Waterborne outbreak of viral gastroenteritis. *Scand. J. Infect. Dis.* **29**, 415–418.

Lee, L. A., Shapiro, C. N., Hargrett-Bean, N., and Tauxe, R. V. (1991). Hyperendemic shigellosis in the United States. A review of surveillance data for 1967–1988. *J. Infect. Dis.* **164**, 894–900.

Lehner, A., Tasara, T., and Stephan, R. (2005). Relevant aspects of *Arcobacter* spp. as potential foodborne pathogen. *Int. J. Food Microbiol.* **102**, 127–135.

Lindquist, R., and Westoo, A. (2000). Quantitative risk assessment for *Listeria monocytogenes* in smoked or gravad salmon and rainbow trout in Sweden. *Int. J. Food Microbiol.* **58**, 181–196.

Little, M. D., Reneau, R. B., and Martens, R. B. (1991). Lime-stabilized and chemically-fixed sewage sludges as lime amendments. *Bioresour. Technol.* **37**, 93–103.

Lytle, D. A., Rice, E. W., Johnson, C. H., and Fox, K. R. (1999). Electrophoretic mobilities of *Escherichia coli* 0157:H7 and wild-type *Escherichia coli* strains. *Appl. Environ. Microbiol.* **65**, 3222–3225.

McFeters, G. A., Bissonette, G. K., and Jezeski, J. J. (1974). Comparative survival of indicator bacteria and enteric pathogens in well water. *Appl. Microbiol.* **27**, 823.

McGinley, K. J., Larson, E. L., and Leyden, J. J. (1988). Composition and density of microflora in the subungual space of the hand. *J. Clin. Microbiol.* **26**, 950–953.

Michel, O. (2003). Role of lipopolysaccaride (LPS) in asthma and other pulmonary conditions. *J. Endotoxin Res.* **9**, 293–300.

Mizgajska, H. (1994). The distribution and survival of eggs of *Ascaris suum* in six different natural soil profiles. *Acta Parasit.* **38**, 170–174.

Modore, M. S., Rose, J. B., Gerba, C. P., Arrowood, M. J., and Sterling, C. R. (1987). Occurrence of *Cryptosporidium* in sewage effluents and selected surface waters. *J. Parasitol.* **73**, 702–705.

Monroe, S. S., Ando, T., and Glass, R. I. (2000). Introduction–human enteric caliciviruses: An emerging pathogen whose time has come. *J. Infect. Dis.* **181**(Suppl. 2), S249–S251.

Moore, A. C., Herwaldt, B. L., Craun, G. F., Calderon, R. L., Highsmith, A. K., and Juranek, D. D. (1993). Surveillance for waterborne disease outbreaks—United States, 1991–1992. *MMWR* **42**, 1–22.

Moore, B. E. (1993). Survival of human immunodeficiency virus (HIV), HIV-infected lymphocytes, and poliovirus in water. *Appl. Environ. Microbiol.* **59**, 1437–1443.

Neilson, J. W., Josephson, K. L., Pepper, I. L., Arnold, R. B., DiGiovanni, G. D., and Sinclair, N. A. (1994). Frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in soil. *Appl. Environ. Microbiol.* **60**, 4053–4058.

Newby, D. T., Pepper, I. L., and Maier, R. M. (2000). Microbial transport. *In* "Environmental Microbiology" (R. Maier, I. L. Pepper, and C. P. Gerba, Eds.), pp. 147–175. Academic Press, San Diego, CA.

Nishijima, S., Namura, S., Kawai, S., Hosokawa, H., and Asada, Y. (1995). *Staphylococcus aureus* on hand surface and nasal carriage in patients with atopic dermatitis. *J. Am. Acad. Dermatol.* **32**, 677–679.

Noble, W. C. (1998). Skin bacteriology and the role of *Staphylococcus aureus* in infection. *Br. J. Dermatol.* **139**(Suppl. 53), 9–12.

NRC (National Research Council). (1996). "Use of Reclaimed Water and Sludge in Food Crop Production." National Academy Press, Washington, DC.

NRC (National Research Council). (2002). "Biosolids Applied to Land: Advancing Standards and Practices," pp. 1–12. National Academy Press, Washington, DC.

Olenchock, S. A. (2001). Airborne endotoxin. *In* "Manual of Environmental Microbiology" (C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney, and L. D. Stetzenbach, Eds.), 2nd ed., pp. 814–826. ASM Press, Washington, DC.

Okazaki, M., Umeda, B., Koide, M., and Saito, A. (1998). Legionella longbeachae pneumonia in a gardener. (in Japanese). *Kansenshogaku Zasshi.* **72**, 1076–1079.

Overgaauw, P. A. (1997). Aspects of toxocara epidemiology: Human toxocarosis. *Crit. Rev. Microbiol.* **23**, 215–231.

Papapetropoulou, M., and Vantarakis, A. C. (1998). Detection of adenovirus outbreak at a municipal swimming pool by nested PCR amplification. *J. Infect.* **36**, 101–103.

Pasquill, F. (1961). The estimation of the dispersion of wind borne material. *Meteorol. Mag.* **90**, 33–49.

Pepper, I. L., Josephson, K. L., Bailey, R. L., Burr, M. D., and Gerba, C. P. (1993). Survival of indicator organisms in Sonoran desert soil amended with sewage sludge. *J. Environ. Sci. Health A* **28**, 1287–1302.

Percival, R., Chalmers, M., Embrey, M., Hunter, P. R., Sellwood, J., and Wyn-Jones, P. (2004). ''Microbiology of Waterborne Disease.'' Elsevier, San Diego, CA.

Pillai, S. D., Widmer, K. W., Dowd, S. E., and Ricke, S. C. (1996). Occurrence of airborne bacteria and pathogen indicators during land application of sewage sludge. *Appl. Environ. Microbiol.* **62**, 296–299.

Rao, V. C., Metcalf, T. G., and Melnick, J. L. (1986). Removal of pathogens during wastewater treatment. *In* ''Biotechnology'' (H. J. Rehm and G. Reed, Eds.), pp. 531–554. VCH, Berlin.

Regli, S., Rose, J. B., Haas, C. N., and Gerba, C. P. (1991). Modeling risk for pathogens in drinking water. *J. Am. Water Works Assoc.* **83**, 76–84.

Rice, E. W. (1999). *Escherichia coli. In* ''Waterborne Pathogens,'' 1st ed., pp. 75–78. AWWA Manual M48, American Water Works Association, Denver, CO.

Rimhanen-Finne, R., Vuorinen, A., Marmo, S., Malmberg, S., and Hänninen, M.-L. (2004). Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. *Soc. Appl. Microbiol.* **38**, 301–305.

Robertson, L. J., Campbell, A. T., and Smith, H. V. (1992). Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. *Appl. Environ. Microbiol.* **58**, 3494–3500.

Robertson, L. J., Smith, H. V., and Paton, C. A. (1995). Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in sewage influent and six sewage treatment plants in Scotland and prevalence of cryptosporidiosis and giardiasis diagnosed in the communities by those plants. *In* ''Protozoan Parasites and Water'' (W. B. Betts, D. Casemore, C. Fricker, H. Smith, and J. Watkins, Eds.), pp. 47–49. Royal Society of Chemistry, Cambridge, UK.

Rohwer, R. G. (1984). Scrapie infectious agent is virus-like in size and susceptibility to inactivation. *Nature* **308**(5960), 658–662.

Rose, J. B., and Gerba, C. P. (1991). Use of risk assessment for development of microbial standards. *Water Sci. Technol.* **24**, 29–34.

Rose, J. B., and Carnahan, R. P. (1992). Pathogen removal by full scale wastewater treatment. Report to Florida Department of Environmental Regulation, Tallahassee, FL.

Rusin, P., Enriquez, C. E., Johnson, D., and Gerba, C. P. (2000). Environmentally transmitted pathogens. *In* ''Environmental Microbiology'' (R. M. Maier, I. L. Pepper, and C. P. Gerba, Eds.), pp. 447–489. Academic Press, San Diego, CA.

Rusin, P., Maxwell, S., Brooks, J., Gerba, C., and Pepper, I. (2003). Evidence for the absence of *Staphylococcus aureus* in land applied biosolids. *Environ. Sci. Technol.* **37**, 4027–4030.

Rylander, R., Lundholm, M., and Clark, C. S. (1983). Exposure to aerosol of microorganisms and toxin during handling of sewage sludge. *In* ''Biological Health risk of Sludge Disposal to Land in Cold Climates'' (P. M. Wallis and D. L. Lehemann, Eds.), pp. 69–78. University of Calgary Press, Calgary, Alberta.

Sabalos, C. M. (1998). ''Detection of enteric viruses in treated wastewater sludge using cell culture and molecular methods.'' (M.S. Thesis), University of Arizona, Tucson, AZ.

Sahlstrom, L., Aspan, A., Bagge, E., Danielsson-Tham, M.-L., and Albihn, A. (2004). Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. *Water Res.* **38**, 1989–1994.

Sano, D., Matsuo, T., and Omura, T. (2004). Virus-binding proteins recovered from bacterial culture derived from activated sludge by affinity chromatography assay using a viral capsid peptide. *Appl. Environ. Microbiol.* **70**, 3434–3442.

Schets, F. M., Engels, G. B., During, M., and de Roda Husman, A. M. (2005). Detection of infectious *Cryptosporidium* oocysts by cell culture immunofluorescence assay: Applicability to environmental samples. *Appl. Environ. Microbiol.* **71**, 6793–6798.

Schijven, J. F., and Rietveld, L. C. (1996). How do field observations compare with models of microbial removal? The Groundwater Foundation 12th Annual Fall Symposium ''Under the microscope: Examining microbes in groundwater,'' September 1996, Boston.

Schwartzbrod, J., and Banas, S. (2003). Parasite contamination of liquid sludge from urban wastewater treatment plants. *Water Sci. Technol.* **47**, 163–166.

Sharif El, N., Douwes, J., Hoe, P. H. M., Doekes, G., and Nemery, B. (2004). Concentrations of domestic mite and pet allergens and endotoxin in Palestine. *Allergy* **59**, 623–631.

Slifko, T. R., Huffman, D. E., and Rose, J. B. (1999). A most probable number assay for enumeration of infectious *Cryptosporidium parvum* oocysts. *Appl. Environ. Microbiol.* **65**, 3936–3941.

Smid, T., Heederik, D., Houba, R., and Quanjer, P. H. (1992). Dust and endotoxin related respiratory effects in the animal feed industry. *Am. Rev. Respir. Dis.* **146**, 1474–1479.

Smith, H., Grimason, T., and Holland, C. (1999). *Ascaris lumbricoides. In* "Waterborne Pathogens." American Water Works Association, Denver, CO.

Sorber, C. A., Moore, B. E., Johnson, D. E., Harding, H. J., and Thomas, R. E. (1984). Microbiological aerosols from the application of liquid sludge to land. *J. Water Pollut. Control Fed.* **56**, 830–836.

Sorvillo, F., Ash, L. R., Berlin, O. G., and Morse, S. A. (2000). *Baylisascaris procyonis*: An emerging helminthic zoonosis. *Emerg. Infect. Dis.* **88**, 355–359.

Stampi, S., De Luca, G., Varoli, O., Zanetti, F. W., Mochmann, H., Richter, U., and Dobberkau, H. J. (1999). Occurrence, removal and seasonal variation of thermophilic campylobacters and *Acrobacter* in sewage sludge. *Zentralbatt Hyg. Unweltmed.* **202**, 19–27.

Stampi, S., Zanetti, F., Crestani, A., and DeLuca, G. (2000). Occurrence and seasonal variation of airborne gram negative bacteria in a sewage treatment plant. *New Microbiol.* **23**, 97–104.

Stelzer, W., Jacob, J., Schulze, E., and Mochmann, H. (1991). The occurrence and survival of *Campylobacter* in sewage sludge. *Zentralbl. Mikrobiol.* **146**, 17–23.

Strachan, N. J., Dunn, G. M., and Ogden, I. D. (2002). Quantitative risk assessment of human infection from *Escherichia coli* O157 associated with recreational use of animal pasture. *Int. J. Food Microbiol.* **75**, 39–51.

Straub, T. M., Pepper, I. L., and Gerba, C. P. (1992). Persistence of viruses in desert soils amended with anaerobically digested sewage sludge. *Appl. Environ. Microbiol.* **58**, 636–641.

Straub, T. M., Pepper, I. L., and Gerba, C. P. (1993a). Virus survival in sewage sludge amended desert soil. *Water Sci. Technol.* **27**, 421–424.

Straub, T. M., Pepper, I. L., and Gerba, C. P. (1993b). Hazards from pathogenic microorganisms in land-disposed sewage sludge. *Rev. Environ. Contamin. Toxicol.* **132**, 55–91.

Straub, T. M., Pepper, I. L., and Gerba, C. P. (1995). Comparison of PCR and cell culture for detection of enteroviruses in sludge-amended field soils and determination of their transport. *Appl. Environ. Microbiol.* **61**, 2066–2068.

Tanner, B. D. (2004). Aerosolization of microorganisms and risk of infection from reuse of wastewater residuals. (Ph.D. Dissertation). University of Arizona, Tucson, AZ.

Tanner, B. D., Brooks, J. P., Haas, C. N., Gerba, C. P., and Pepper, I. L. (2005). Bioaerosol emission rate and plume characteristics during land application of liquid Class B biosolids. *Environ. Sci. Technol.* **39**, 1584–1590.

Taylor, M. R. (2001). The epidemiology of ocular toxocariasis. *J. Helminthol.* **75**, 109–118.

Teunis, P. F. M., Nagelkerke, N. J. D., and Haas, C. N. (1999). Dose-response models for infectious gastroenteritis. *Risk Anal.* **19**, 1251–1260.

Thomaz-Soccoi, V., Paulino, R. C., Castro, E. A., and Andreoli, C. V. (1997). Helminth eggs viability in sewage and biosolids sludge in Curitiba, Parana, Brazil. *Arquivos de Biologia E Technologia* **40**, 829–836.

Voss, J. G. (1975). Effects of an antibacterial soap on the colony of aerobic bacterial flora of human skin. *Appl. Microbiol.* **30**, 551–556.

Wang, X. W., Li, J., Guo, T., Zhen, B., Kong, Q., Yi, B., Li, Z., Song, N., Jin, M., Xiao, W., Zhu, X., and Gu, X. (2005). Concentration and detection of SARS coronavirus in sewage

from Xiao Tang Shan Hospital and the 309th Hospital of the Chinese People's Liberation Army. *Water Sci. Technol.* **52**, 213–221.

Ward, R. L., McFeters, G. A., and Yeager, J. G. (1984). Pathogens in sludge: Occurrence, inactivation and potential for regrowth Sandia Rept. DAND 83–0667, TTC–0428, UC–71 Sandia Natl. Labs, Albuquerque, NM.

Watkins, J., and Sleath, K. P. (1981). Isolation and enumeration of *Listeria* monocytogenes from sewage, sewage sludge, and river water. *J. Appl. Bacteriol.* **50**, 1–9.

Wei, S., Walsh, P., Huang, R., and To, S. S. (2000). 93G, a novel sporadic strain of hepatitis E virus in South China isolated by cell culture. *J. Med. Virol.* **61**, 311–318.

Welbourn, E., Champion, R. H., and Parish, W. E. (1976). Hypersensitivity to bacteria in eczema. I. Bacterial culture, skin tests and immunofluroescent detection of immunoglobulins and bacterial antigens. *Br. J. Dermatol.* **94**, 619–632.

Westrell, T. C., Schonning, C., Stenstrom, T. A., and Ashbolt, N. J. (2004). QMRA (quantitative microbial risk assessment) and HACCP (hazard analysis and critical control points) for management of pathogens in wastewater and sewage sludge treatment and reuse. *Water Sci. Technol.* **50**, 23–30.

Wolk, D. M., Johnson, C. H., Rice, E. W., Marshall, M. M., Grahn, K. F., Plummer, C. B., and Sterling, C. R. (2000). A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. *Septata intestinalis. Appl. Environ. Microbiol.* **66**, 1266–1273.

Yates, M. V. (1994). Monitoring concerns and procedures for human health effects. *In* "Wastewater Reuse for Golf Course Irrigation," pp. 143–171. CRC Press, Boca Raton, FL.

Zaleski, K. J., Josephson, K. L., Gerba, C. P., and Pepper, I. L. (2005a). Potential regrowth and recolonization of *Salmonella* and indicators in biosolids and biosolid amended soil. *Appl. Environ. Microbiol.* **71**, 3701–3708.

Zaleski, K. J., Josephson, K. L., Gerba, C. P., and Pepper, I. L. (2005b). Survival, growth, and regrowth of enteric indicator and pathogenic bacteria in biosolids, compost, soil, and land applied biosolids. *J. Residuals Sci. Technol.* **2**, 49–63.

This page intentionally left blank

Advances in Crop Water Management Using Capacitive Water Sensors

A. Fares and V. Polyakov

Natural Resources and Environmental Management Department,
College of Tropical Agriculture and Human Resources,
University of Hawaii-Manoa, Honolulu, Hawaii 96822

Advances in microelectronics during the last decades resulted in the development of several dielectric-based soil water monitoring techniques, that is, time-domain reflectometry, single and multisensor capacitance probe (MCP) systems. These techniques have simplified the real-time determination of water content at fine spatial and temporal scales. In this chapter, single and MCP systems will be referred to as capacitance. Because of their relatively low cost and ease of operation, capacitance has enjoyed widespread acceptance among researchers, growers, and consultants. This chapter gives

43

an overview of the principle of operation of capacitance soil water content measuring systems. Installation and calibration procedures of these systems were also covered. The applications of capacitance are diverse. They have been used extensively as an essential part of many irrigation scheduling programs for different crops. We used real-time logging capabilities of capacitance to give more detailed information about plant water uptake, effective rainfall, and also to determine some soil physical properties. Results of these types of studies demonstrate that considerable improvement in efficient water use can be made by collecting high-resolution soil water content data in the soil, around the crop. Despite their success, the capacitance system showed some temperature and salinity effects for different soil types. Further research is needed to eliminate these salinity and temperature effects if these capacitance systems are to take their place as some of the leading soil water monitoring sensors.   © 2006, Elsevier Inc.

I.   INTRODUCTION

Demands on water resources worldwide are increasing as the world population keeps growing and quality of living keeps improving in many countries. It is expected that in the next decade several countries in the arid and semiarid areas of the globe will be under water scarcity or stress (Yang *et al.*, 2003). As the major water user, irrigated agriculture is expected to make substantial changes to optimize its water use. Water is critical for optimal growth and production. Optimum amounts of water at the right time allow plants to grow and produce at their best with little or no negative impact to the environment.

Several water management techniques have been developed, tested, and used with different levels of success. For over 40 years since its introduction, neutron moisture gauge, often referred to as neutron scattering (NS), proved to be very popular as a research and teaching tool with the scientific community, but also for application in a wide range of agricultural, environmental, and engineering practices. Its widespread use resulted partly from the ease, speed, and the nondestructive nature of its measurement as compared with conventional gravimetric methods. Since the release of its first prototype, NS has seen several improvements such as weight and size reductions and the introduction of more efficient detectors that also used safer radioactive sources. However, despite these improvements, safety regulations requiring costly licensing and training of users and considerable regulation have caused the NS method to remain expensive to maintain and difficult or impossible to use in some situations, particularly for unattended monitoring (Evett and Steiner, 1995).

Advances in microelectronics and emergence of high-quality, low-cost, high-frequency oscillators have made capacitance sensors popular *in situ* soil water content monitoring devices and consequently made automated capacitance soil water sensors more affordable (Fares and Alva, 2000). Capacitance sensors have been used to measure soil water content in a wide range of soil types by researchers and growers in Australia (Buss, 1993; Fares *et al.*, 2004b) and the United States (Baumhardt *et al.*, 2000; Fares and Alva, 2000) for various applications such as irrigation scheduling (Girona *et al.*, 2002) and waste water disposal (Olden *et al.*, 2002).

II. CAPACITANCE SOIL WATER CONTENT MEASURING SYSTEMS

A. PRINCIPLE OF OPERATION

The water molecule is a dipole due to the presence of two partial positive charges on the hydrogen sides and a negative charge on the oxygen atom of the same molecule. Materials composed of molecules that are permanently polarized, that is, water molecule, usually have large dielectric constants, also known as dielectric permittivity. The dipolar nature of the water molecule provides bulk water unique electrical properties that have long been the target of measurement of water in substances such as soil (Topp and Ferre, 2002). In the absence of any external electric field, water molecules are in random thermal motion. However, when an external electric field is applied, the charged molecules align themselves with the electric field where the positive charges of each molecule are in the direction of the applied field and the negative charges oppose the field. An internal electric field, which is opposite in direction of the external electric field, will result. Consequently, a reduction of the overall electric field and the overall potential occurs.

Capacitance has been used to determine dielectric permittivity of different homogeneous and mixed media. Capacitance is defined as the ability of two conductors to store a charge Q when a potential V is applied across them. The relationship between the capacitance, C $(L^{-2} T^4 M^{-1} I^2)$, of a capacitor with a geometric factor, g (L), and the relative permittivity, ε_r $(L^{-3} T^4 M^{-1} I^2)$, is defined as the following:

$$C = \varepsilon_r \varepsilon_o D \tag{1}$$

where ε_o is the permittivity in vacuum (8.5 pF m^{-1}). If the capacitor is formed by two parallel plates then $D = A/d$, where A is the area of the plates (L^2) and d

is the distance between the two plates (L) filled with the dielectric of permittivity ε.

A typical capacitance soil water sensor consists of an inductor, L, and a capacitor connected to circuitry that oscillates at a frequency that is dependent on the values of L and the electrode-soil capacitor (Starr and Paltineanu, 2002). Inductor L is set by the electronic circuitry; the frequency of oscillation depends only on variations of capacitance. Several representations of the oscillation frequency of capacitance sensors have been presented (Dean, 1994; Kelleners *et al.*, 2004; Starr and Paltineanu, 2002). Assuming a resonant frequency in an oscillator circuit that includes the soil, this resonant frequency is represented as follows:

$$F = \left(2\pi \sqrt{C_s \frac{C_p C_m}{C_p + C_m}} \right)^{-1} \tag{2}$$

where C_m, C_p, and C_s are the capacitance of the medium, plastic access tube (for capacitance sensor with access tubes), and capacitance due to stray electric fields, respectively.

Complex permittivity is a property that describes both polarization and absorption of energy. The real part is related to polarization while the imaginary part is related to energy absorption. The relative permittivity ε_r is defined as follows:

$$\varepsilon_r = \varepsilon' + i[\varepsilon'' + \sigma/(\omega\varepsilon_o)] \tag{3}$$

where ε' and ε'' are the real and imaginary (electric loss) parts, respectively, of the permittivity; σ is the zero-frequency conductivity; ω is the angular frequency; ε_o is the free-space permittivity; and i is the imaginary part of a complex number. At frequencies between 100 MHz and 3–4 GHz, ε'' is much smaller than ε' (Ledieu *et al.*, 1986) and, therefore, the measured permittivity is called the apparent permittivity ε_a (Topp *et al.*, 1980). The apparent permittivity represents the real part of the complex relative permittivity at the highest frequency (Heimovaara *et al.*, 1994). Assuming that the dielectric properties of soil water are the same as that for bulk water, several dielectric mixing models have been developed to estimate the dielectric properties of wet soils, including a theoretical de Loor model (de Loor, 1964), a semiempirical model by Birchak *et al.* (1974), and an electric circuit model that relates the sensor frequency to the permittivity of the medium (Kelleners *et al.*, 2004).

Capacitance soil water sensors operate at a narrow band frequency and use dielectric constant (K), of the soil–water–air mixture to estimate soil water content. The K of water (78.54 at 22°C) is large compared to those of the soil matrix (<10) and air (1), and, thus, dominates the dielectric permittivity of the air–soil–water mixture. A change in soil water content will strongly influence

the K of soil. However, great variability of the K of soil minerals (4–9) (Robinson, 2004) and dry plant tissue (1–4) makes it necessary to calibrate these sensors for a particular soil (Baumhardt *et al.*, 2000) and, if practical, for each soil horizon. In addition, K is a function of the ratio of free water to that of bound water, soil temperature, bulk density, and water salinity, especially at low sensor frequencies (Paltineanu and Starr, 1997). In most cases, the relationship between the multisensor capacitance probe (MCP) output and volumetric soil water content (θ_v) is a three-parameter power function (Fares *et al.*, 2004b; Paltineanu and Starr, 1997); however, linear approximation was also used for some soils (Gaudu *et al.*, 1993). The nonlinearity of the relationship between MCP output and θ_v is partially attributed to clay-bound water, which has a behavior that is different from that of free water under the influence of electromagnetic waves (Bridge *et al.*, 1996; Wang, 1980).

B.   EQUIPMENT DESIGN

There are a number of capacitance probe designs, which differ from each other by electrode configuration and geometry (Fig. 1), range of operating frequencies (50–150 MHz), and ease of use and accuracy. Conceptually, capacitance sensor systems can be subdivided into single and multisensor
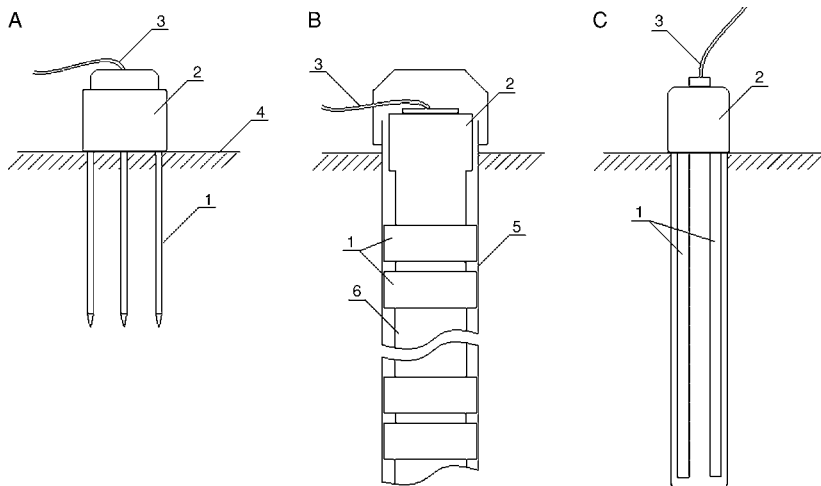

Figure 1 Schematic design of three capacitance probes with different types of electrodes: (A) rod-type electrodes with direct soil contact, (B) ring electrodes in tube housing, and (C) flat electrodes sealed in plastic. 1—Electrode, 2—electronic circuitry, 3—cable, 4—soil surface, 5—PVC access tube, and 6—circuit board.

systems. They also have different electrode designs: rod, flat, or cylindrical types.

1. Single-Sensor System

The rod-type probe consists of an input/output cable, probe body, and a sensing head (Fig. 1). The sensing head of rod electrode sensors consist of an array of four rods (1 signal and 3 shield rods). The cable provides connection for a power supply and for an analog signal output. The probe body contains an oscillator, a specially designed internal transmission line, and measuring circuitry within a waterproof housing. The outer three rods are connected to instrument ground and form an electrical shield around the central, signal rod. The sphere of influence of this type of sensor is contained in the space between the rods and the measurement is integrated along their length. They can be inserted in the soil surface or into augured holes or, alternatively, in the wall of a trench (which is then backfilled). Among the disadvantages are soil disturbance and possibility of soil compaction or formation of air gaps during the insertion into the ground.

An example of parallel rod-type sensor is Theta Probe, which measures volumetric soil moisture content with 1% accuracy (Delta-T-Devices, 1999). It requires 5–15 V DC at 20 mA power supply. Its operating frequency is 100 MHz.

Flat electrode capacitance sensor, that is, ECH_2O (Decagon, Pullman, WA) consist of copper electrodes positioned in one plane and sealed in epoxy-impregnated fiberglass (Fig. 1). The electrodes have no direct contact with the soil, and the electromagnetic field generated by the electrodes extends through the fiberglass and into the soil surrounding the probe. These sensors are easy to install and use. The probe averages the volumetric water content over the entire length of the probe. If water content measurement in a narrow soil layer is required, the probe needs to be installed horizontally. The 2 cm zone of influence extends from the flat surface of the probe, decreasing with distance. The sensor has little sensitivity at the extreme edges. The ECH_2O probes are well suited for use at shallow depth; however, soil temperature fluctuations at such depth could affect the measurement. The temperature dependence of the probes is 0.003 cm^3 cm^{-3} $^\circ C^{-1}$ (Campbell, 2003). The sensor has very low power requirement (2–5 V DC at 3–7 mA). ECH_2O probes allow using cable of up to 78-m length.

2. Multisensor Probe System

The principles of operation and design of the MCP were described in detail by Buss (1993), Paltineanu and Starr (1997), and Fares and Alva

(2000). Examples of such systems are EasyAg® 50 (Sentek, 2003) and C-probe (Agrilink, 2005). EasyAg® 50 system consists of multisensor probes and data logger, capable of operating 32 sensors (8 probes with 4 sensors each) simultaneously. An individual probe consists of an access tube, printed circuit board, and four capacitance sensors (Fig. 1). Access tube is a 60-cm long special PVC pipe with external and internal diameters of 32 and 28 mm, respectively. The diameter of the capacitance ring was such that allowed the sensor to move freely inside the access tube but with minimum air gap between the rings and access tube wall.

Each capacitor sensor consists of two metal rings mounted on the circuit board at the distance 10, 20, 30, and 50 cm from the top of the access tube. These rings are a pair of electrodes, which form the plates of the capacitor with the soil acting as the dielectric in-between. The plates are connected to an oscillator, consisting of an inductor and a capacitor. The oscillating electrical field is generated between the two rings and extends into the soil medium through the wall of the access tube (99% of the reading is taken within 10-cm radius around the sensor axis). The capacitor and the oscillator form a circuit, and changes in dielectric constant of surrounding media are detected by changes in the operating frequency. The capacitance sensors are designed to oscillate in excess of 100 MHz inside the access tube in free air. The output of the sensor is the frequency response of the soil's capacitance due to its soil moisture level.

C. Installation

Installation of capacitance sensors should begin with careful selection of the site to conduct soil water monitoring. Site selection could be conceptually subdivided in two stages: macro- and microzone selection. Soil type and soil hydrological characteristics are spatially variable properties and may differ significantly within short distances. The aim of soil water monitoring, however, is to provide the data, which can be used for management decisions applicable for the whole field using limited number of sensors. Thus, macrozone selection refers to the selection of one or several locations on the field characterized by soil type, crop, and management practice dominant for the area and have typical topography. Microscale zone selection is aimed to determine the position of the sensor in relation to individual plants, irrigation delivery points (drip line, sprinkler, and so on), and various surface and soil anomalies. When installing the capacitance sensors it is recommended to avoid proximity of unusually large or sick plans, areas free of plants, locations with compacted or disturbed soil, microdepressions, and proximity to foreign objects (metal posts, elements of irrigation

equipment, and so on). These factors may not only affect the sensor response but also alter soil water dynamics, making the soil around the sensor unrepresentative of the studied area. When drip line or furrow irrigation is used, soil water content varies significantly across the crop row. In such cases, it is recommended to use two or more sensors at the locations of projected maximum (under the drip line) and minimum soil moisture (between the rows).

In order to make correct measurement of soil water content by capacitance technique, the integrity of the studied object (soil) during the installation must be preserved. Special attention must be given to avoid air gaps between the sensor or its access tube and the soil. Considering almost two order of magnitude difference between the dielectric constant of water and air (80:1), the presence of even small air gaps may significantly affect the collected data.

Capacitance sensors installation procedure differs depending on the type of the sensor used. However, the general guideline is that soil disturbance should be minimized and good contact between probes or their access tubes should be ensured. When installing access tubes, it is a good practice to cover the soil surface where the installation will be conducted with boards or plywood to prevent compaction.

Rod sensors, such as ML2x (Fig. 1), are carefully pushed into the ground in one steady motion, avoiding any sideway pressure. This ensures that no air gap is formed between the rods and the soil. It is also important to avoid compaction of soil by the probe housing. If installation below the soil surface is needed, the electrodes are installed horizontally in the wall of soil pit at the desired depth and the soil pit is backfilled. When digging the pit, each soil horizon needs to be extracted and kept separately. When the pit is filled, different horizons are placed back in the same order as they were extracted, thus, preserving the morphology of the soil profile. In addition, the soil needs to be compacted similar to its original density. Although the soil in the pit has no direct impact on the sensor reading, the disturbance may create preferential water flow and affect local soil water dynamics.

The installation of sensors with flat electrodes, such as ECH$_2$O (Fig. 1), is similar to that of rod-type sensors. If the soil allows, the electrode is pushed into soil surface or side of the soil pit taking precautions as previously described. If the soil is too dense, a thin cut in the ground, deep enough to accommodate the full length of the electrode, is made using shovel or blade. The electrode is placed into the cut and the shovel is inserted into the soil parallel to the electrode 10–15 cm away from it. The soil in-between is gently pushed toward the electrode closing the air gap between the electrode and the surrounding soil. Care must be taken to avoid excessive compaction.

Capacitance sensors with ring-type electrodes are installed on the field using an access tube (Fig. 1). The electrodes do not come in direct contact with the soil. The procedure may vary slightly, depending on the accessories provided by the manufacturer and the probe type. For small diameter sensor a vertical hole in the soil is made using a standard soil auger with the diameter slightly smaller than that of the access tube. To direct the auger vertically and prevent it from wobbling, a stabilization plate is used. The stabilization plate is a piece of metal with an opening through which auger is inserted. The stabilization plate is secured to the ground by four metal pins. It also protects the edges of the hole and prevents it from collapsing. It is recommended to insert the auger to the required depth (slightly larger than the length of the access tube) in one take. If the soil is dense, the auger might need to be removed and cleaned several times before the desired depth is reached. Before the insertion of the access tube, a cutting ring with sharp edge tapered inside is attached to the tube's lower end. The cutting ring has the diameter equal to that of the access tube. When the access tube is pushed into the hole, the cutting ring shaves excess soil from the walls of the hole, providing tight fit and good contact of the tube and soil. If the soil is dry, a small amount of water is added using a squirt bottle. When the tube is installed in place, the stabilization plate is removed. Capacitance electrode rings mounted on the circuit board plate are inserted into the access tube, cables are connected, and the tube is closed with watertight cap. Cable opening in the cap is sealed with silicon glue. For large diameter sensors, auguring is conducted from inside of the access tube, while the tube is being gradually pushed into the ground. When the installation of the access tube is complete, its lower end is sealed with a rubber plug.

D.   DATA LOGGING AND DISPLAYING

Frequent sampling of soil moisture is the key to identify trends in soil water dynamics. Modern capacitance probe systems often consist of network of sensors, representing different sites on the monitored area and different depths within soil profile. Each of the major manufacturers of capacitance soil water content monitoring systems developed their proprietary software that have been used to manage their system, display data, and in some cases analyze the collected data. Capacitance probe systems include specialized software utilities used for hardware administration and communication. These utilities include:

1. Database manager used to view and manage logger configuration, create databases, organize existing databases, and view existing databases configuration.

2. Download manager utility provides basic logger diagnostics, setup and management functions, connects between PC or PDA and data logger, retrieves raw data from the logger, and downloads it to PC hard drive for further use.
3. Display utility displays real-time moisture reading, creates soil moisture graphs, helps to visualize the depth of irrigation or rainfall event, rates of infiltration into deep soil profiles, and the rates of water consumption by plants.
4. Remote connection manager used to operate wireless data retrieval hardware.
5. Workspace manager utility manages all windows associated with a workspace and opens data files.

Figure 2 shows water content measured using an MCP system at 10, 20, 30, and 50 cm below the soil surface in a citrus grove at the University of Florida Avalon research site. This type of graph is produced by many of the commercial softwares that are sold with different capacitance systems, that is, IrriMax 6.1 (Sentek, Pty., Ltd., Stepny, South Australia). This period was a rain-feed period; daily rainfall events are shown on the same graph as
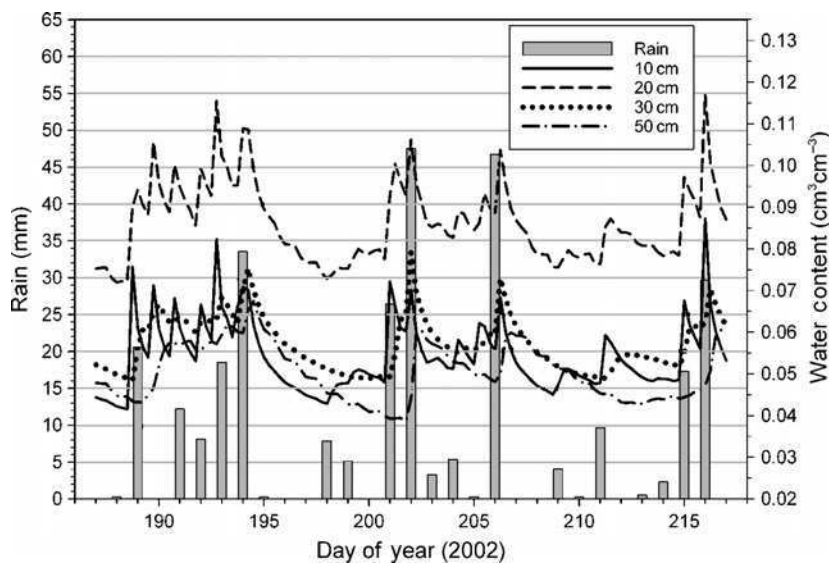

Figure 2 Water content measured using an MCP system at 10, 20, 30, and 50 cm below the soil surface in a citrus grove at the University of Florida Avalon research site.
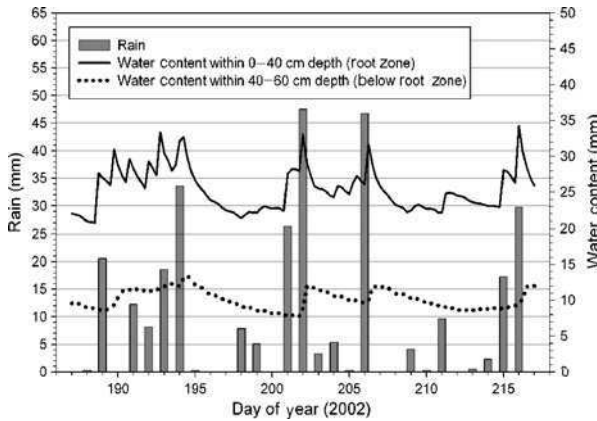
Figure 3 Cumulative water content within and below the root zone in a citrus grove at the University of Florida Avalon research site.

bar graph. Daily rainfall intensity varied between less than 2 mm to over 45 mm. Changes of soil water content over time at the various depths are the result of the rainfall events, plant water uptake, and water redistribution below the root zone. The soil of this research site is Candler fine sand with low field capacity, permanent wilting point, and water-holding capacity of 0.09, 0.015, and 0.075 cm^3 cm^{-3}, respectively. The soil water dynamics are more pronounced in the top two depths (10 and 20 cm) than in lower 30 and 50 cm depths.

Cumulative water content within and below the root zone is displayed in Fig. 3 along with the daily rainfall events for the same period shown earlier. Water content in the root zone shows more dynamics than that below the root zone. Water content dynamics below the root zone is an indicator of the excess water losses below this citrus grove under sandy Florida soils. This type of data were used to calculate drainage losses below citrus groves in central Florida (Fares and Alva, 1999, 2000).

E. CALIBRATION

Although default calibration equations are often provided by the manufacturers that are based on calibration in a variety of soils, capacitance sensors may require custom calibration for a number of reasons. It has been reported (Dobson *et al.*, 1985; Wang, 1980) that soil type affects the

apparent K of soil, and, therefore, θ_v measurements based on K. The primary cause of difference between soils is usually attributed to the effects of solid–liquid interactions at the solid surface, which restrict the rotational freedom of adsorbed water molecules (Seyfried and Murdock, 2001). This water, called bound water, is considered to have K much lower than that of the free water (Dobson *et al.*, 1985), and its amount is expected to positively correlate with the soil surface area. Consequently, with the increase of clay content in the soil, the proportion of bound to free water will also increase, shifting sensor measurement. Hence, θ_v is underestimated by default equation at low moisture range, where the impact of bound water is more profound.

Paltineanu and Starr (1997) corroborated that in addition to clay amount, differences in soil mineralogy, especially 2:1 clays, could affect MCP instrument calibration. In other words, large surface areas of 2:1 clays affect the bound water and corresponding bulk permittivity (Bridge *et al.*, 1996; Wraith and Or, 1999). Similar conclusion was reported by Baumhardt *et al.* (2000) in Olton soil that has mixed mineralogy, which includes the 2:1 clay, montmorillonite, as the dominant clay mineral.

MCP requires normalization procedure. The normalization procedure is necessary for the MCP to overcome variability between individual sensors. Normalization ensures that the measurements obtained from different sensors are comparable, and the sensors within MCP are interchangeable and can be replaced without the loss of data continuity. The ring-type sensors are normalized by placing an access tube into a water bath and in the air at $20\,^{\circ}C$ and recording the respective frequency readings for each sensor. These two media represent two extreme conditions, where the capacitor oscillates at its maximum (air) and minimum (water) frequencies.

The frequency of the capacitor in the soil is used to determine a scaled frequency (SF) value using the following equation:

$$SF = (F_a - F_s)(F_a - F_w)^{-1} \tag{4}$$

where F_a, F_w, and F_s represent the sensor readings placed in its access tube in air at room temperature ($22\,^{\circ}C$), in a bath of water at room temperature, and in the field soil, respectively. The value of the SF varies between 0 and 1 depending on air–water–soil mixture of the medium; thus, the higher the SF the higher the water content is. The SF is then used as input into a calibration equation to determine the corresponding soil water content. The SF is calculated when the data are downloaded from the logger and then converted into θ_v using a default or user-specified calibration equation. Similar normalization can be conducted for other types of sensors if greater precision, consistency of multiple reading, and comparability of data obtained by different sensors are required.
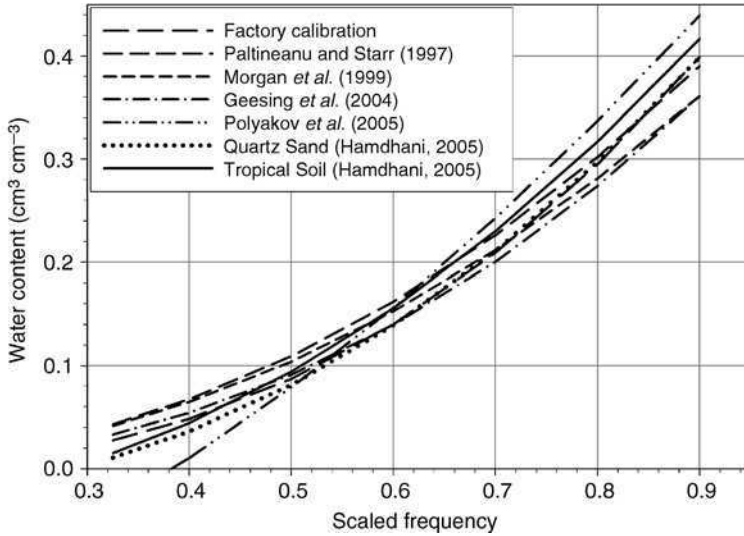
Figure 4 Calibration results of MCP on various soils and quartz sand as compared to the equation provided by the manufacturer.

Capacitance sensors have been calibrated in the field (Fares *et al.*, 2004b; Morgan *et al.*, 1999) and laboratory (Baumhardt *et al.*, 2000; Hamdhani, 2005; Paltineanu and Starr, 1997; Polyakov *et al.*, 2005) conditions; however, limited number of calibration exercises were conducted using weathered tropical soils where bound water effect is present (Wu, 1998). In most cases, the relationship between the SF and θ_v has been described by two- (Morgan *et al.*, 1999; Paltineanu and Starr, 1997) and three-parameter (Baumhardt *et al.*, 2000; Fares *et al.*, 2004b) power models (Fig. 4). However, linear approximation was also used for some soils (Gaudu *et al.*, 1993). The nonlinearity of the relationship between MCP output and θ_v is partially attributed to clay-bound water, which has a behavior that is different from that of free water under the influence of electromagnetic waves (Bridge *et al.*, 1996; Wang, 1980). However, the nonlinear relationship in sand has also been observed (Paltineanu and Starr, 1997). Several studies (Baumhardt *et al.*, 2000; Fares *et al.*, 2004b) proved the superiority of the three-parameter model as compared to the two-parameter model:

$$\theta_v = a + b(SF)^c \qquad (5)$$

where *a*, *b*, and *c* are fitting coefficients.

Figure 5 Calibration results of MCP on highly weathered Hawaiian soil (Ewa silty clay loam) and quartz sand in laboratory conditions as compared to the equation provided by the manufacturer (Reprinted from Polyakov *e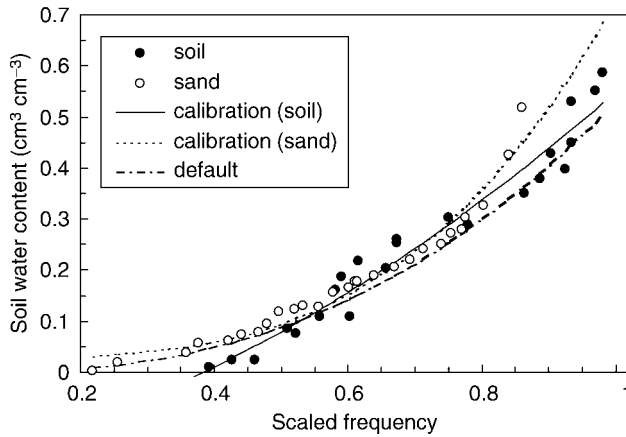t al.* (2005). Calibration of a capacitance system for measuring water content of tropical soil. *Vadoze Zone J.* **4**, 1004–1010).

Polyakov *et al.* (2005) developed separate calibration equations for several media with increasing degree of dielectric complexity: sand, soil, and soil with shrinking-swelling clays (Fig. 5). There were minimal differences between the default calibration curve and that of the sand over lower range of the water content (0–0.3 cm^3 cm^{-3}). The default manufacturer calibration equation underestimated the water content in the sand over the upper range of the water content. This is consistent with results reported by Morgan *et al.* (1999). They demonstrated that in Florida sandy soils (sand ≥96%), soil water content was underestimated by the default calibration of the MCP in the low soil water content range. However, Geesing *et al.* (2004) reported mixed results in coarse-textured soil; they found that the default calibration equation of MCP overestimated the soil water content for $\theta_v \geq 0.25$ cm^3 cm^{-3}. In the lower water content range ($\theta_v \leq 0.13$ cm^3 cm^{-3}) MCP underestimated water content.

Polyakov *et al.* (2005) reported that differences between θ_v estimates using the default calibration and the measured θ_v were more pronounced for Ewa soil than those for sand (Fig. 5). The default calibration underestimated the water content for $\theta_v \geq 0.1$ cm^3 cm^{-3} and overestimated the water content for $\theta_v \leq 0.1$ cm^3 cm^{-3}. Geesing *et al.* (2004) reported that for silty-loam soil, default calibration of their tested MCP generally underestimated the actual soil water content.

III.   APPLICATION OF CAPACITANCE AS WATER MANAGEMENT DEVICES: IRRIGATION SCHEDULING FOR DIFFERENT CROPS

Maintenance of adequate soil water content through the crop-growing season is necessary for optimum plant growth and yield. In most cases, soil water content is at optimum level only for a short period during the growing season; hence, irrigation is needed to maintain adequate soil water availability. The purpose of managed irrigation is to optimize water spatial and temporal distribution, promote crop growth and yield, and increase economic returns.

Irrigation scheduling is considered as a decision-making process used by irrigators to decide when to irrigate their crops and determine the appropriate quantity of water to apply. However, irrigation scheduling has received limited attention among farm operators. Pleban and Israeli (1989) indicated two possible reasons for this: (1) the programs were oriented toward research and for use by professionals, and (2) the programs were looking at the scheduling problem from the point of view of the crop and the academic researcher and not the farmer. The authors suggest that in addition to these two obstacles, water pricing and environmental policies often are not adequate to provide sufficient incentive or regulation to improve the management of water on the farm. Furthermore, some moisture-measuring devices, such as time-domain reflectometry (TDR) and neutron probes, remain outside the range of affordable equipment by typical growers in many locations or do not have the capacity to log soil moisture status in real time (Tyndale-Biscoe and Malano, 1995). In this respect, the use of capacitance-based systems has a considerable advantage, and their use by practitioners has been growing.

When water is applied on the soil surface, assuming little or no surface runoff, a portion of water is utilized by plants or retained by the soil, however, excess water drains through the vadoze zone into the groundwater, which contributes to aquifer recharge. Irrigation best management practices are designed to (1) optimize plant water and nutrient uptake, (2) minimize water and nutrient leaching below the root zone, (3) minimize nonpoint source pollution of groundwater, and (4) reduce production costs associated with water and nutrient losses by leaching.

Supply of water to crops must be based on a clear understanding of the soil water dynamics. Using low-volume irrigation systems, such as drip irrigation, it is possible to schedule small but frequent irrigation events to maintain an optimum water content in the root zone, while decreasing water losses below this depth. Tracing of water budget requires the monitoring of all water inputs and outputs, including rainfall, irrigation, drainage, runoff,

evapotranspiration (ET), and changes in soil water storage in near-real time. For many years, growers have relied on plant visual symptoms as indication of need for irrigation. Some plant species, such as beans, show sufficient color change when under moisture stress, which can be used as a basis to schedule irrigation. However, many crops do not show consistent visual symptoms of low moisture stress until the plants suffered severe stress effects. Furthermore, the soil moisture status may change rapidly so that watering may be required before visual symptoms are noticeable. Growth processes cease in many crops before visual wilting occurs, so that by the time there are indications of the need for irrigation, yield reduction may already have occurred (Smajstrla and Harrison, 1982).

The use of capacitance sensors for irrigation scheduling may be well described using a case study of citrus irrigation management in Florida. The EnviroSCAN® (Sentek Pty. Ltd., Stepney, South Australia) capacitance sensors were used for continuous monitoring of the soil water content of young citrus trees (Fares and Alva, 2000). The experiment, using 4-year-old Hamlin orange trees in Calder fine sand was conducted on the Citrus Research and Education Center, University of Florida, Lake Alfred, FL. The trees were irrigated using a low-volume, under-the-tree irrigation system with one emitter per tree with a 0.05 m^3 h^{-1} delivery rate. The depth of maximum root activity for these trees was experimentally determined to be at 40 cm and the root system was found within 7.3 m^2 area around the trunk. Soil water content through the soil profile was monitored using probes with capacitance-based sensors at 10-, 20-, 40-, 70-, and 110-cm depth, installed on each treatment. The 110-cm sensor is well beyond the active root zone of most crops, however, it provided valuable information as to how much water, either as rains or irrigation, is required for leaching to occur.

The target refill points for optimal irrigation scheduling were based on an evaluation of the allowable soil moisture depletion within the rooting depth of 40 cm. The available soil moisture (ASM) content was determined as follows:

$$ASM = \theta_{FC} - \theta_{PWP} \tag{6}$$

where θ_{FC} is the volumetric water content (cm^3 cm^{-3}) at field capacity, and θ_{PWP} is the volumetric soil water content (cm^3 cm^{-3}) at wilting point.

For Candler fine sand θ_{FC} and θ_{PWP} were 0.09 and 0.015 cm^3 cm^{-3}, respectively. Hence, the ASM for the top 40-cm of effective rooting depth was 30 mm. Optimal citrus production requires that ASM is depleted no more than 33% during the period from February to May and no more than 67% during the remaining part of the year. The goal of each irrigation event was to deliver adequate amount of water to replenish the deficit in the top 40-cm to field capacity. This target point is defined as the ''full point,''

which was equivalent to 36 mm for the target depth of irrigation. The water content in the soil profile within and below the rooting zone was calculated for the three wetted area treatments.

Using near real-time information provided by the capacitance probes it, was possible to maintain the water content in the root zone of the 7.3 m^3 wetted area treatment above 33% depletion refill point and minimize water losses below root zone for almost the entire period (Fig. 6). This type of graph helps in determining how much irrigation to apply. Management decisions are made by determining where the soil moisture graph is positioned in relation to the refill point and full point. The refill point is positioned on the graph as a point that could result in water stress due to lack of moisture. The full point is set at a value below saturation to avoid excess moisture and potential leaching of nutrients.

Volumetric soil water contents measured using the capacitance probes were used to calculate the water content for the entire profile. Figure 6 shows the mean water content (of three capacitance probes randomly placed in the field) for the 0- to 40-, 40- to 110-, and 0- to 110-cm depth profile. Irrigation was scheduled based on the recommended refill points depending on the tree growth stages. Accordingly, the soil water content in the root zone was maintained within 33% depletion of the ASW during the critical period from February to June and at 67% during the rest of the year. From Fig. 6 it is evident that the water content in the root zone was maintained within the target refill points during the entire growing season, except for few occasions during February to June when the water content in the root zone dropped slightly below the refill point just before the subsequent irrigation event. The sharp increase and decrease in the water content following each irrigation or rain event clearly demonstrates the low water-holding capacity and high hydraulic conductivity of this soil.

The increase in water content in the soil immediately below the root zone is due to water drainage from the root zone. During the first 90 days of the year, the water content below the root zone varied around 40 mm, except on 19 January when it increased to 60 mm as a result of 42 mm of irrigation applied as a precautionary measure to protect against forecasted freezing temperatures. In April, the water content below the root zone showed large increases in response to heavy rainfall events. This was due to a net total rainfall of 174 mm, which was nearly fivefold greater than the long-term average. May and June were drier than the average. This explains the decrease in the water content below the root zone as compared with its level during April. The remaining portion of the year was in the rainy season, and an unusually wet fall resulted in excess water drained below the root zone.

Capacitance sensors were used for irrigation scheduling on Creamgold onions in Australia (Muldon *et al.*, 1999) on self-mulching clay soils. The sensors were installed at 10-cm intervals up to 100-cm depth on 1.5-m beds
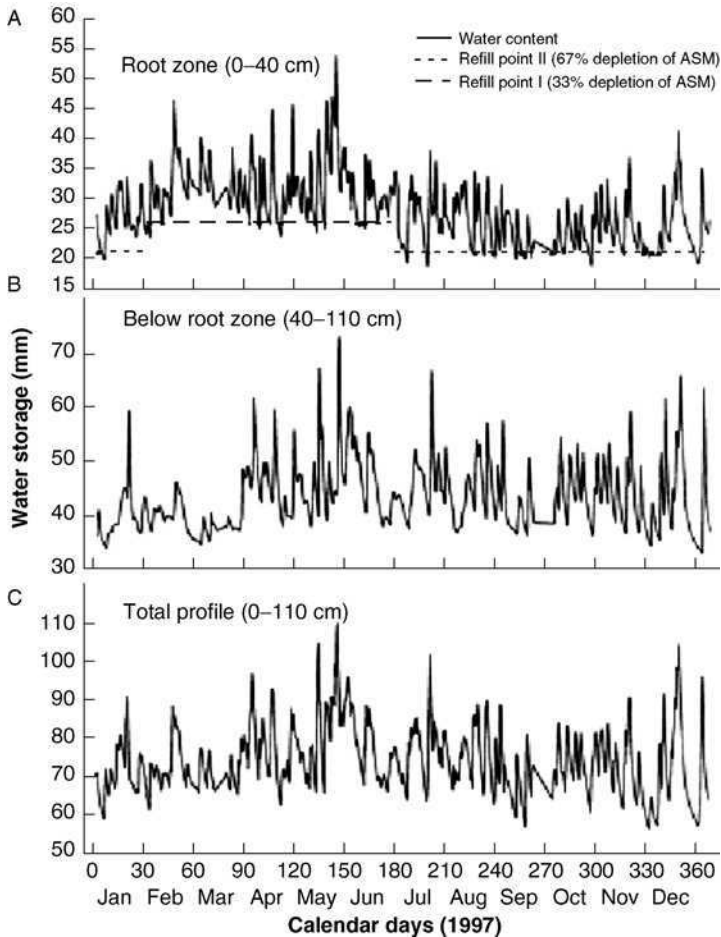
Figure 6 Depth-integrated soil water content (A) within and (B) below the root zone, and for (C) the entire monitored soil profile depth during 1997. The refill points indicate the soil water content at which irrigation was scheduled to replenish the water deficit (Reprinted from Fares, A., and Alva, A. K. (2000). Soil water components based on capacitance probes in a sandy soil. *Soil Sci. Soc. Am. J.* **64**, 311–318).

where onions were planted in five rows and furrow-irrigated. For this soil type, the ASM was 0.09 cm^3 cm^{-3}. Onions extracted water from the depth of up to 50 cm below which the soil remained at field capacity. Three irrigation events were applied when water content was well below the refill point. It was estimated that out of 4×10^6 L ha^{-1} of water applied, 2.4×10^6 L ha^{-1}

was used by the crop, producing a crop yield of 65 t ha^{-1}. Further reduction of water losses was possible by using more frequent irrigation events with less volume of water per event.

Alva *et al.* (2003) evaluated the application of capacitance probes for real-time monitoring of soil moisture both within and below the root zone of a potato crop in a sandy soil in the Pacific Northwest of the United States. They reported a good correlation of soil moisture content measured by capacitance probes and that measured using destructive soil sampling.

An automatic soil water station (ASWS) using capacitance sensors was used to monitor soil water content and irrigation scheduling of potato crops planted in ridges and beds in the United Kingdom (Robinson, 1999). The soil sensors were installed at 0.15-, 0.25-, and 0.5-m depth below the top of the ridge/bed. Hourly data were collected so that a high temporal resolution data set could be constructed in order to increase conceptual understanding of hydrological processes at a scale appropriate to the crop. The author demonstrated that the water available to the crop was very limited; effectively between 0.25 and 0.08 cm^3 cm^{-3}. In addition, a deficit of no greater than 15 mm was allowed to develop in the soil so that the surface of the potato is kept moist to prevent infection by scab. The ASWS data helped to establish that most of the water bypassed the potatoes planted in ridges as irrigation water applied to the crop from a boom irrigator was shed off the ridges infiltrating in the furrows. A soil water deficit built up inside the ridge was not replenished by irrigations. A second early potato crop planted in beds was more successful at capturing water as the flat bed increased water infiltration around the crop.

Moons and Gilmore (2001) reported a number of positive results on capacitance probes for irrigation scheduling of potato in Manitoba. The growers confirmed the probes' accuracy through their use in commercial production. They were able to avoid gross error in irrigation scheduling by managing their soil moisture within the refill and full points. As a result, water was used more efficiently throughout the growing season.

An integrated real-time irrigation scheduling system has been developed for some crop in southeastern Australia (Malano *et al.*, 1996). The system comprises a soil moisture monitoring device, a medium-term weather forecast, and a decision support system to assist irrigators in making irrigation scheduling and water ordering decisions. Three soil moisture monitoring devices, including capacitance sensors, were tested over two irrigation seasons for accuracy and reliability, and during this time were used to assist in the irrigation scheduling task.

Thompson *et al.* (2004) developed dynamic protocols for drip-irrigated vegetable crops grown in greenhouse-covered soils using MCP. Melon, tomato, and pepper were grown sequentially. The manufacturer's recommended protocols were applied to melon, using a single EnviroSCAN system

probe located 6 cm from the plant and 10 cm from the emitter, with four sensors at different depths up to 40 cm. Compared to a tensiometer-managed treatment, total irrigation volume applied, fruit production, and soil matric potential (10-cm depth) were very similar. These protocols were suggested for this and similar drip-irrigated horticultural systems.

These works demonstrate that considerable improvement to efficient water use can be made by collecting high-resolution soil water content data in the soil, around the crop in real or near-real time. Field trials incorporating this style of technology to understand soil water processes provide a comprehensive way of studying crop water response.

IV. DETERMINATION OF SOIL WATER PHYSICAL PROPERTIES

A. FIELD SOIL WATER STORAGE

The storage capacity of a soil profile (soil water storage capacity, SWSC) is the depth of water required to raise a shallow water table to the soil surface (Nachabe *et al.*, 2004). The concept of SWSC as introduced by Nachabe *et al.* (2004) is fundamental to many hydrological processes, including surface runoff by saturation excess, expansion and contraction of wetlands, and estimation of the length of an overland flow plane. They introduced and tested a model to estimate SWSC using simultaneous observations of shallow water table fluctuations and soil moisture in a shallow, sandy soil (hyperthermic Aeric Alaquods). The water table at their selected sites fluctuated between a shallow depth and the soil surface during the summer, allowing frequent observation of surface inundation and profile storage. They also used the following equation: $\text{SWSC} = Ad^B + Cd + D$ to adequately describe the variability of SWSC with d, depth to the water table and where the parameters A, B, C, and D are easily derived from basic physical properties of the soil horizons, including porosity and water retention. They further showed that the SWSC can be significantly limited by the capillary fringe above the water table, encapsulated air (the volume of air trapped under positive pressure beneath the water table), or the presence of a clay pan at shallow soil depths. The capillary fringe had some influence on SWSC in this sandy soil, but encapsulated air as high as 11.0% of the soil volume was observed at the site. Encapsulated air reduced the available soil storage and resulted in a rapid rise in water table. Ignoring encapsulated air significantly underestimated soil profile storage. Storage results including and excluding air encapsulation were compared as a function of water table depth.

MCP was also employed to monitor soil water dynamics in a sandy soil within and below the root zone of corn (Starr and Timlin, 2004). Temporal variability in soil moisture was recorded during soil water recharge and high ET periods October to April and May to September, respectively. All water parameters (infiltration, net storage, drainage, and crop water uptake) were the lowest during the recharge period, and lower under no-till (NT) management than plow-till (PT).

The relationship between the soil water content and the soil water suction, defined as the soil water release curve, is a fundamental part of the characterization of the hydraulic properties of the soil (Klute, 1986). Knowledge of the soil water release curves is important for effective irrigation of crops. Soil water release curves and saturated hydraulic conductivities can be determined in the laboratory, using disturbed and undisturbed soil cores (Klute, 1986; Klute and Dirksen, 1986) or in the field (*in situ* method) as described by Bruce and Luxmoore (1986) and Elrick *et al.* (1984). The *in situ* field method of determination of the soil water release curve is known as the instantaneous profile method. This method gives more realistic results of the field hydraulic conductivity and soil water release curves than the laboratory measurements. Before the introduction of real-time measurement of soil water contents with MCP, this method used to be labor intensive and requires considerable time and efforts (Bruce and Luxmoore, 1986).

The instantaneous profile method has been used by several researchers on different soil types (Bruce and Luxmoore, 1986; Dane and Puckett, 1992; Davidson *et al.*, 1969; Gardner, 1970; Giesel *et al.*, 1970). It requires frequent and simultaneous measurements of soil water content and matric potential for given depth of soil profile under conditions of drainage. The automatic acquisition of matric potential data is usually facilitated by pressure transducers, while corresponding soil water content is monitored by neutron probe. The use of capacitance probes for the instantaneous profile method was limited (Fares *et al.*, 2000). A pioneer study to determine soil water release curves using capacitance probes at five soil depths was conducted in Candler fine sand in Florida (Fares *et al.*, 2000).

Water content and pressure potentials for five depths were monitored at frequent time intervals using capacitance probes and tensiometers. The suction varied from 0 at soil saturation, in the beginning of the experiment, to 80 cm at a soil water content of $0.05 \text{ cm}^3 \text{ cm}^{-3}$ (Fig. 7). This was due to rapid water drainage from large pores, which constitute the majority of the pores in sandy soil. The water drainage from fine pore space requires more energy (Parlange *et al.*, 1998), which is indicated by leveling of the retention curve at suction higher than 40 cm (Fig. 7). Results of this study (Fares *et al.*, 2000) compared well with the data reported by Sodek *et al.* (1990) for the same soil in laboratory conditions. The authors demonstrated that the
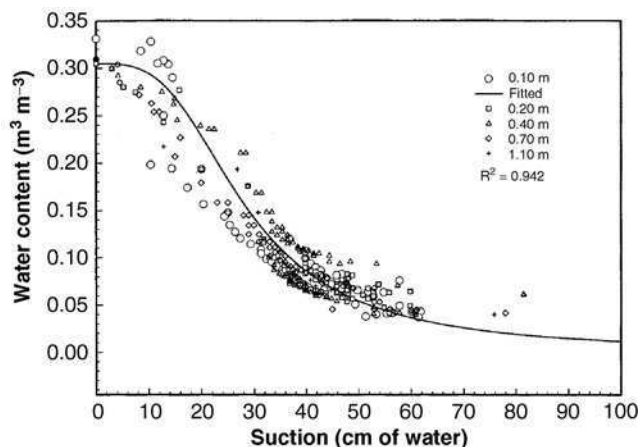
Figure 7 Volumetric water content and soil water matric head for the entire soil profile (0–110 cm) of Candler fine sand (Reprinted from Fares *et al.* (2000). Estimation of soil hydraulic properties of a sandy soil using capacitance probes and guelph permeameter. *Soil Science* **165**, 768–777).

capacitance probe is a practical tool that can be effectively used to determine *in situ* soil water characteristic curves at different soil depths.

Morgan *et al.* (2001) compared tensiometers and resistance blocks matric potential values to soil water content values from capacitance sensors calibrated gravimetrically in a Florida fine sandy soil. The effective range of the capacitance sensors in fine sandy soils allowed the authors to obtain water release curves in the range between 5 and 20 kPa. Soil water potential values for both sensors (tensiometers and resistance blocks) were within 2 kPa of the mean for each sensor. Change in the soil matric potential was similar over the range of water content 0.04–0.08 cm^3 cm^{-3}. Retention curves for the two sensors were different by 4 kPa at 0.04 cm^3 cm^{-3} water content. Soil water retention curves obtained using capacitance sensors and tensiometers and resistance blocks were shown to be robust and convenient techniques.

B. FIELD UNSATURATED HYDRAULIC CONDUCTIVITY

Because tensoimeters have a limited range of operation, field measurements of hydraulic conductivity (k), θ_v, and h are often combined with approximate analytical functions (van Genuchten, 1980) to estimate the k–θ_v–h relationship over the wide range needed for irrigation design and scheduling and for water–solute–crop growth modeling.
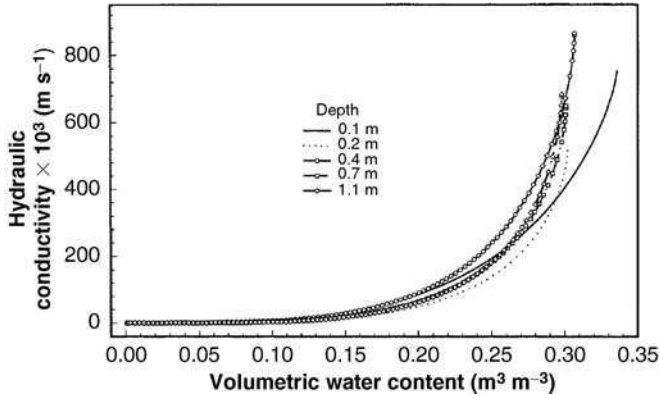
Figure 8 Hydraulic conductivity as a function of volumetric water content for the 10-, 20-, 40-, 70-, and 110-cm soil profile of a Candler fine sand during water redistribution (Reprinted from Fares *et al.* (2000). Estimation of soil hydraulic properties of a sandy soil using capacitance probes and guelph permeameter. *Soil Science* **165**, 768–777).

The relationship between the unsaturated hydraulic conductivity and the soil water content was derived by van Genuchten (1980) and is expressed as following:

$$k(\theta) = k_s S_e^{1/2} \left[1 - \left(1 - S_e^{1/m} \right)^m \right]^2 \tag{7}$$

where $S_e = (\theta - \theta_r)/(\theta_s - \theta_r)$; θ_s (cm^3 cm^{-3}) is the water content at saturation; θ_r (cm^3 cm^{-3}) is the residual water content; θ (cm^3 cm^{-3}) is the water content at which k is being calculated; m is a fitting parameter; and k_s (cm day^{-1}) is the saturated hydraulic conductivity.

Fares *et al.* (2000) used capacitance probes, tensiometers, Guelph permia- meter, and van Genuchten hydraulic function [Eq. (7)] to determine unsatu- rated hydraulic conductivity in a fine sand soil. Figure 8 obtained using capacitance sensors data shows that the hydraulic conductivity of sandy soil decreased exponentially as the volumetric water decreased to 0.1 cm^3 cm^{-3}. The rate of increase in $k(\theta)$ was small at low water content but increased sharply as it approached saturation (Fig. 8). This rate of decrease of the hydraulic conductivity increased sharply between water content of 0.1 cm^3 cm^{-3} and the residual water content level. Thus, the hydraulic conductivities dropped from their maximum at saturation values (7.06 × 10^{-5} and 11.57 × 10^{-5} m s^{-1}) to 9.72 × 10^{-8} to 4.87 × 10^{-7} m s^{-1} at 0.1 cm^3 cm^{-3} volumetric soil water content. The decrease in $k(\theta)$ was greater as the volumetric soil water content decreased below 0.1 cm^3 cm^{-3} to the permanent wilting point.

By combining the Guelph permiameter and analytical fitting software RETC (van Genuchten *et al.*, 1991), it was possible to estimate $k(\theta)$ at different soil water contents and, consequently, soil matric potential. Results of this study demonstrate that capacitance sensors are practical tools that can be used effectively to determine unsaturated hydraulic conductivity over a wide water content range. These results are very helpful for irrigation management and water losses determination.

C.   Spatial and Temporal Distributions of Soil Physical Properties

Gish *et al.* (2002) employed MCP to investigate the influence of subsurface stratigraphies on subsurface soil water movement up to 1.8-m depths. They reported that the shape of restrictive layers at the interface of different textures within the soil profile affects the relationship between soil depth and water storage capacity. Soil moisture decreased with increasing depth in profiles immediately above concave or flat restrictive layers, while the inverse relationship was observed above bowl-shaped restrictive layers. They also used MCP data to document matrix flow along topographical gradients.

Nachabe *et al.* (2005) investigated spatial and temporal variabilities in total soil moisture (TSM) and ET as measured with MCP in a mixed vegetation system of pasture with a riparian forest buffer. Short-term temporal variability was determined to be a function of balance between surface water discharge and ET with soil water storage increasing overnight when ET approximated zero and dropping during the day as a result of high ET. Over the long-term, temporal variability was also a function of ET with higher soil moisture during the recharge period when ET was low.

V.   USE OF MCP TO CALCULATE DIFFERENT FIELD WATER CYCLE COMPONENTS

A.   Plant Water Use

Plant water use and ET are used interchangeably in this section of the chapter. Fares and Alva (1999, 2000) used real-time soil water content data collected through an MCP system to optimize citrus irrigation under Florida ridge soil. The data provided by the MCP were used with daily rainfall, irrigation, soil water characteristic curves, and effective rooting depth to determine the components of citrus water balance. Daily ET and

drainage below the root zone were calculated at three different locations. Their daily ET varied seasonally, ranging from 0.4 mm day^{-1} in January to 5.0 mm day^{-1} in July and August. These daily ET values were well within the daily ET values reported for citrus trees in Florida.

Nachabe *et al.* (2005) introduced a method to estimate ET in shallow water table environments under Florida conditions. This method measures the diurnal fluctuations in TSM above the water table to estimate (1) the net lateral and vertical subsurface flux in the aquifer and (2) ET from the vegetation cover. They showed that in a hillslope discharge zone, the net lateral subsurface flux was calculated from the recovery rate of soil moisture between midnight and 0400 h. ET was then estimated from a daily water balance in a soil column that included the water table. They tested this method on two vegetation covers, a pasture in a groundwater recharge area and a riparian zone with woody vegetation in a groundwater discharge area. An MCP carrying eight sensors was used in each area to estimate the total soil water content. During their study period, the measured water table depth fluctuated between soil surface and a depth of 1.2 m, allowing observation and estimation of the TSM in a soil column that included the water table. The results of this investigation support another hypothesis that, in humid, shallow water table environments, ET demand may be supported by adjacent ecosystems. This method provided reasonable results for the two landscapes investigated and was able to capture the variability of ET in heterogeneous vegetation covers. This is a relatively inexpensive alternative to characterize ET within regionally heterogeneous but microhomogenous landscapes. This tested method can be easily adapted to other soil types with shallow water table. Another advantage of this method is that ET can be successfully estimated without detailed knowledge of soil hydraulic properties, subsurface flow patterns, or vegetation characteristics.

Diurnal fluctuation in ET was observed to be the driving force behind temporal variability under corn (Starr and Paltineanu, 1998). Spatial variability under the monoculture was related to variability in soil management. In areas under NT management, soil water storage was higher than that under PT management.

Paltineanu and Starr (2000) quantified the row and interrow soil water dynamics under multiple annual no-tillage and plow-tillage corn experiments. Real-time soil water dynamics was studied for a 3-year period with MCPs. Small-scale, spatially dependent variation in real-time soil water dynamics was largely caused by cultural practices that resulted in localized changes and patterns of preferential water flow. Smaller rainfall events (<15 mm), summer rains, resulted in significantly increased soil water storage in the 5- to 55-cm soil depths for the NT in-row position, compared with the other NT interrow positions, and compared with the plow-tillage in-row position.

These results show that using a real-time soil water monitoring system with MCPs placed at the in-row and interrow positions of corn versus real-time data of incoming rainfall could highly improve the understanding of real-time water dynamics phenomena in the soil–plant–atmosphere continuum. Coupling measured plant canopy effects on water redistribution by stemflow versus throughfall with real-time measures of water inputs by rainfall or irrigation, and soil water dynamics with depth and time can lead to better understanding and prediction of water and chemical penetration in soils.

B. Drainage Below the Root Zone

Darcy's equation is the standard equation implemented in all numerical water flow models that has been used to calculate water movement in porous medium, that is, drainage losses below certain depth. Fares and Alva (1999, 2000) used this tool along with MCP real-time data and soil physical properties to calculate daily drainage rates at three different locations within a citrus grove in central Florida. Their results showed that daily drainage varied considerably through the two-year-study period (1996–1997). The mean drainage values for 1997 were as high as 47 mm. In general, the daily drainage rates were low during January to March, which represents the dry months when the optimal irrigation is critical for the trees. The rainfall in April 1997 was fivefold greater than the long-term average and accordingly resulted in several large drainage events. Greater than 40% of the annual cumulative drainage occurred during October to December, which were unusually wet months. The mean cumulative annual drainage in 1997 was 890 mm or 47% of the total water input (irrigation + rainfall = 1810 mm). Furthermore, they reported that 82% of the cumulative annual drainage was contributed by rainfall. Irrigation based on monitoring soil water content using capacitance probes minimized water drainage below the root zone in a system where rainfall contributed substantially to drainage.

C. Effective Rainfall

Effective rainfall (ER) is defined as useful or utilizable rainfall. Some of the rainfall may be unavoidably lost due to the combined effect of rainfall intensity, frequency, and amount. Just as total rainfall varies, so does the amount of ER. The useful portion of rainfall is stored and supplied to the plant for its use. Water-regulating agencies often require accurate estimates

of the components of the crop water budget in order to fairly allocate irrigation water resources to growers (Obreza and Pitts, 2002). This means that ER needs to be estimated.

Several methods have been used to calculate ER. Technical Release No. 21 (TR-21) has been used worldwide to calculate ER and predict irrigation requirements. Improvement in real-time soil water monitoring sensors provided a good opportunity to test the accuracy of the TR-21 in estimating ER. Obreza and Pitts (2002) used a spreadsheet to develop an analytical model that implements the TR-21 equation to calculate ER. They computed ER as

$$ER = P - RO - DP \qquad (8)$$

where P is precipitation, RO is run-off, and DP is deep percolation (precipitation that moves below the root zone).

Fares *et al.* (2004a) used two MCP systems to investigate shading and irrigation effects on spatiotemporal distribution of rain, plant water uptake, and water content under mature Hamlin orange trees grown in a Florida sandy soil. Most citrus groves in Florida are irrigated with microsprinklers. These systems do not wet the entire grove floor. Hence, ER in citrus groves with microsprinkler systems is spatially and temporarily variable. Soil water contents under the trees in three directions (north, south, and west of the trunk), at three locations (0.9, 1.8, and 3.0 m away from the trunk), and at 0.1, 0.2, 0.4, and 0.8 m below the soil surface (Fares *et al.*, 2004a). Rain gauges were installed under and outside citrus tree canopy between two adjacent tree rows close to the MCP. The soil water status in both irrigated and nonirrigated zones was monitored in real time. This allowed determining ER within and outside tree canopy. There were significant differences in water content dynamics between the irrigated and nonirrigated areas of the citrus grove. Results of this study showed that 100% of the 55.9 mm of rainfall that occurred was effective in the nonirrigated area of the groves; however, only 63% was ER for the irrigated area under the tree canopy (Fig. 9). The major factors that contributed to low ER in the irrigated area were rainfall interception by the canopy and higher water content in this zone due to irrigation.

Paltineanu and Starr (2000) evaluated spatial variability in canopy interception and plant water uptake under rain-fed corn grown on a Maryland silt loam soil. These investigators found that the amount of intercepted rainfall redistributed to the ground as stemflow was 10–30 times greater than throughfall. However, the ratio of stemflow to throughfall was lower under heavy rainfall events and later in the season when drooping leaves reduced the amount of intercepted rainfall that was redirected to the stem. The influence of intercepted rainfall redistribution on spatial variation in soil water storage was evaluated using MCP and determined to be largely a
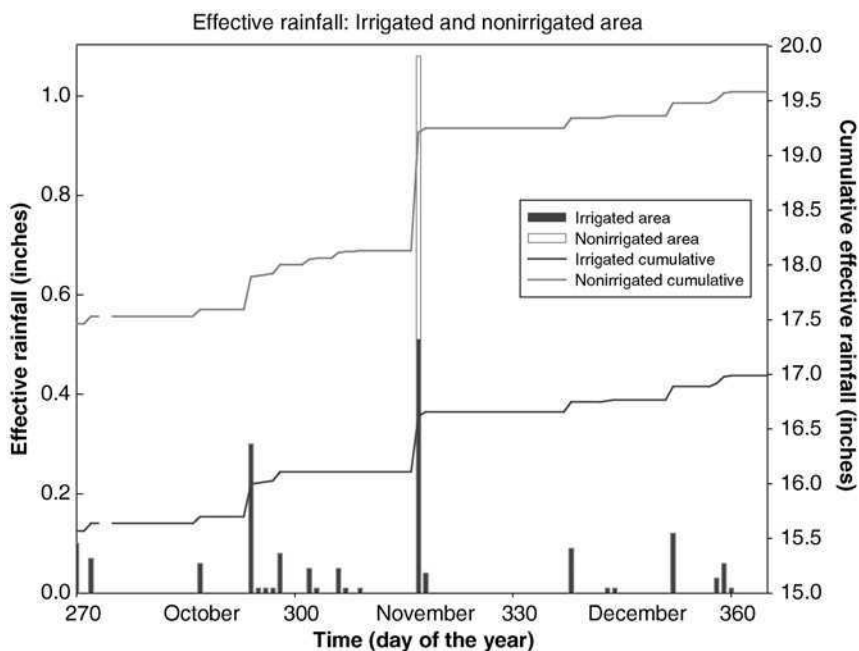
Figure 9   Daily and cumulative effective rainfall (ER) for the irrigated and nonirrigated areas of citrus grove (Fares *et al.*, 2004).

function of tillage. Under low rainfall events (<5 mm), almost all rainfall was redistributed to the soil within row. However, changes in soil water storage were detected only in plots under NT, but not under PT, management, indicating that water did not infiltrate beyond 5 cm where the first sensor was positioned in the tilled plots. This was attributed to the generally higher moisture measurements under NT plots. Following heavy rainfall events, surface and subsurface movement of excess stemflow to interrow areas limited lateral spatial variability. However, infiltration rate and depth were greater in NT than PT plots; this phenomenon was also attributed to higher moisture measurements under NT plots.

VI.   EFFECT OF FLUCTUATION OF SOIL TEMPERATURE AND SOIL SALINITY ON THE PERFORMANCE OF MCP

The effect of soil temperature on capacitance and TDR sensors has been reported for different soil types (Baumhardt *et al.*, 2000). In 5–45°C range,

the dielectric constant of water decreases with the increase of temperature at an approximate rate of $0.36°C^{-1}$ (Weast, 1986). In addition, a small decrease of capacitance frequency, which means an apparent increase in water content, may be attributed to the temperature effects on the sensor's circuitry (Dean *et al.*, 1987). Seyfried and Murdock (2001) reported negative effect of temperature on measured value of θ_v for sand, while for various soils the relationship was positive, resulting in large apparent θ_v variation across a 40°C temperature change. Kuraz (1982), studying temperature effects on fine sand with a portable capacitance sensor at different water contents, reported an increase in the frequency of the capacitance sensor with increasing sand temperature from 15 to 30°C. The observed trend was much more evident at higher water contents. Baumhardt *et al.* (2000) observed cyclical fluctuation in θ_v induced by similar soil temperature fluctuations of 15°C as estimated by MCP and TDR. Wraith and Or (1999) demonstrated experimentally that temperature affects soil permittivity by changing the physical properties of water bound near the surface of 2:1 clay minerals. Furthermore, Yu *et al.* (1999) established theoretically that changes in temperature of the mineral soil fraction have little impact on soil permittivity. Baumhardt *et al.* (2000) attributed θ_v fluctuations indicated by both TDR and MCP to temperature-dependent fluctuations in the soil permittivity.

However, it is likely that the dielectric constant for soil water is less than that for free water because of the interactions between soil water and the soil surface (Hasted, 1972). The dielectric constant of water is temperature dependent (Weast, 1986). It decreases with increasing temperature; thus, it is reasonable to assume that the dielectric constant of wet soils behaves in a similar way, leading to errors in the measurements if temperature effects are not accounted for (Persson and Berndtsson, 1998). More research has been conducted on temperature effects on TDR than on capacitance probes. There have been conflicting reports of any significant temperature effects on water content measurements using TDR (Halbertsma *et al.*, 1995; Pepin *et al.*, 1995; Persson, 1997; Seyfried and Murdock, 2001; Wraith and Or, 1999) and capacitance probes (Baumhardt *et al.*, 2000; Dean *et al.*, 1987; Hamdhani, 2005; Mead *et al.*, 1995; Paltineanu and Starr, 1997; Polyakov *et al.*, 2005).

Paltineanu and Starr (1997) studied the effect of air and water temperatures on capacitance readings. They found that the calculated effects of temperature on water content across the range 10–30°C was in the order of the root mean square error (RMSE) for their calibration curve. Mead *et al.* (1995) reported significant salinity and temperature effects on soil water measurements using MCP. Several hypotheses were postulated to explain the temperature effect on soil water content. The increase of the dielectric constant in some soils was attributed to the release of bound water at high temperature to free water in soils with high specific surfaces (Or and

Wraith, 1999; Pepin *et al.*, 1995; Wraith and Or, 1999). Results obtained by Wraith and Or (1999) show that the higher the surface area and the lower the water content, the more likely it is to observe an increase in TDR-measured bulk dielectric constant. Persson (1997) suggests three different explanations for the increase in the dielectric constant with temperature: (1) contributions of the imaginary component of the dielectric constant [Eq. (3)], (2) overestimation of the dielectric constant due to increasing soil electric conductivity, and (3) the release of bound water. High salt content in the soil has previously been shown to lead to overestimation of the water content (Noborio, 2001; White *et al.*, 1994).

VII. CONCLUSIONS

Success of capacitance soil water monitoring devices has generated interest in using this technology in areas other than irrigation scheduling. Capacitance determines the soil water content using the dielectric constant of the soil–air–water mixture. This chapter covered several topics relevant to capacitance, including theory, calibration, different applications of this technology, and obstacles facing capacitance soil water content sensor. Some of the challenges that have been facing this industry includes: (1) Further research to study and correlate the real-time components of water balance in the soil–plant–atmosphere system over large areas will enhance our understanding and predictive capability of water and chemicals in agricultural systems. (2) Most of the existing capacitance sensors are affected by soil temperature and salinity, thus, limit their spread. High soil salinity and temperature are real problems in arid and semiarid environments, where soil water sensors are needed to optimize the use of the limited water resources. Efforts are needed to eliminate or at least minimize these negative effects of soil salinity and temperature on the performance of capacitance sensors. (3) Expanding needs are increasing in the area of spatial and temporal analysis of natural resources requiring the development of different sensors, that is, water content and nutrient-specific sensors. Improved capacitance sensors have the potential to help meet these needs.

ACKNOWLEDGMENTS

REFERENCES

Agrilink, M. H. (2005). C-Probe Agrilink Holdings Pty Ltd., Thebarton, Australia.

Alva, A. K., Collins, H. P., Fraisse, C., and Boydston1, R. A. (2003). Evaluation of enviroscan capacitance probes for monitoring soil moisture in center pivot irrigated potatoes. *J. Appl. Irrig. Sci.* **38**, 93–110.

Baumhardt, R. L., Lascano, R. J., and Evett, S. R. (2000). Soil material, temperature, and salinity effects on calibration of multisensor capacitance probes. *Soil Sci. Soc. Am. J.* **64**, 1940–1946.

Birchak, J. R., Gardner, C. G., Hipp, J. E., and Victor, J. M. (1974). High dielectric constant microwave probes for sensing soil moisture. *IEEE* **62**, 93–98.

Bridge, B. J., Sabburg, J., Habash, K. O., Ball, J. A. R., and Hancock, N. H. (1996). The dielectric behaviour of clay soils and its application to time domain reflectometry. *Aus. J. Soil Res.* **34**, 825–835.

Bruce, R. R., and Luxmoore, R. J. (1986). Water retention: Field methods. *In* "Methods of Soil Analysis. Part 1: Physical and Mineralogical Methods" (A. Klute, Ed.), pp. 663–686. ASA, Madison, WI.

Buss, P. (1993). The use of capacitance based measurements of real time soil water profile dynamics for irrigation scheduling. *In* "Proc. Natl. Conf. Irrig. Assoc., Australia and Natl. Committee Irrig. Drain., Lauceston, Tasmania," May 17–19. Irrig. Assoc. of Aust., Homebush, NSW, Australia.

Campbell, C. S. (2003). Response of ECH$_2$O soil moisture sensor to temperature variation. DECAGON Application Note. Decagon Devices, Inc. Pullman, WA.

Dane, J. H., and Puckett, W. E. (1992). Field soil hydraulic properties based on physical and mineralogical information. *In* "Methods for Estimating the Hydraulic Properties of Unsaturated Soils" (M. T. van Genuchten, Ed.). University of California, Riverside, CA.

Davidson, J. M., Nielsen, D. R., and La Rue, M. E. (1969). Field measurement and use of soil water properties. *Water Resour. Res.* **5**, 1312–1321.

de Loor, G. P. (1964). Dielectric properties of heterogeneous mixtures. *Appl. Sci. Res.* **B3**, 479–482.

Dean, T. J. (1994). "The IH capacitance probe for measurement of soil water content," Rep. No. 125. Institute of Hydrology, Wallingford, UK.

Dean, T. J., Bell, J. P., and Baty, A. J. B. (1987). Soil moisture measurement by an improved capacitance technique.1. Sensor design and performance. *J. Hydrol.* **93**, 67–78.

Delta-T-Devices (1999). ThetaProbe soil moisture sensor. User Manual. Delta-T Devices Ltd., Cambridge, UK.

Dobson, M. C., Ulaby, F. T., Hallikainen, M. T., and Elrayes, M. A. (1985). Microwave dielectric behavior of wet soil.2. Dielectric mixing models. *IEEE T. Geosci. Remote* **23**, 35–46.

Elrick, D. E., Reynolds, W. D., Lee, D. M., and Clothier, B. E. (1984). The "Guelph Permiameter" for measuring the field saturated hydraulic conductivity above the water table: 1. Theory, procedures and application. "Proceedings of Canadian Hydrological Symposium," Quebec City.

Evett, S. R., and Steiner, J. L. (1995). Precision of neutron-scattering and capacitance type soil-water content gauges from field calibration. *Soil Sci. Soc. Am. J.* **59**, 961–968.

Fares, A., and Alva, A. K. (1999). Estimation of citrus evapotranspiration by soil water balance. *Soil Sci.* **164**, 302–310.

Fares, A., and Alva, A. K. (2000). Soil water components based on capacitance probes in a sandy soil. *Soil Sci. Soc. Am. J.* **64**, 311–318.

Fares, A., Alva, A. K., Paramasivam, S., and Nkedi-Kizza, P. (1997). Soil moisture monitoring techniques for optimizing citrus irrigation. *Trends Soil Sci.* **2**, 153–180.

Fares, A., Alva, A. K., Nkedi-Kizza, P., and Elrashidi, M. A. (2000). Estimation of soil hydraulic properties of a sandy soil using capacitance probes and guelph permeameter. *Soil Sci.* **165**, 768–777.

Fares, A., Parsons, L. R., Obreza, T. A., and Morgan, K. T. (2004a). Spatial and temporal plant water use and rain inputs as affected by citrus canopy and micro sprinkler irrigation system. "Proceedings of the Irrigation Association Annual Meeting," Tampa, FL.

Fares, A., Buss, P., Dalton, M., El-Kadi, A. I., and Parsons, L. R. (2004b). Dual field calibration of capacitance and neutron soil water sensors in a shrinking-swelling clay soil. *Vadoze Zone J.* **3**, 1390–1399.

Gardner, W. R. (1970). Field measurement of soil water diffusivity. *Soil Sci. Soc. Am. Proc.* **34**, 832.

Gaudu, J. C., Mathieu, J. M., Fumanal, J. C., Bruckler, L., Chanzy, A., Bertuzzi, P., Stengel, P., and Guennelon, R. (1993). Soil moisture measurement using a capacitive probe. *Agronomie* **13**, 57–73.

Geesing, D., Bachmaier, M., and Schmidhalter, U. (2004). Field calibration of a capacitance soil water probe in heterogeneous fields. *Aust. J. Soil Res.* **42**, 289–299.

Giesel, W., Lorch, S., and Tenger, M. (1970). Water flow calculation by means of gamma adsorption and tensiometer field measurement in the unsaturated soil profile. *In* "Isotop Hydrology." Int. Atomic Energy Agency, Vienna.

Girona, J., Mata, M., Fereres, E., Goldhamer, D. A., and Cohen, M. (2002). Evapotranspiration and soil water dynamics of peach trees under water deficits. *Agr. Water Manag.* **54**, 107–122.

Gish, T. J., Dulaney, W. P., Kung, K.-J. S., Daughtry, C. S. T., Doolittle, J. A., and Miller, P. T. (2002). Evaluating use of ground-penetrating radar for identifying subsurface flow pathways. *Soil. Sci. Soc. Am. J.* **66**, 1620–1629.

Halbertsma, J., van den Elsen, E., Bohl, H., and Skierucha, W. (1995). Temperature effects on TDR determined soil water content. *In* "Proceedings of the Symposium" (L. W. Petersen and O. H. Jacobsen, Eds.), Time domain reflectometry applications in soil science. Research Centre Foulum, Denmark, Sep. 16, 1994. SP report no. 11, Vol. 3. Danish Institute of Plant and Soil Sci., Lyngby, Denmark.

Hamdhani, O. H. (2005). Capacitance sensor: Calibration, temperature effects and performance in establishing optimal irrigation management of some vegetable crops in Hawaii. (Master Thesis). p. 68. University of Hawaii at Manoa.

Hasted, J. B. (1972). Liquid water: Dielectric properties. *In* "Water, a Comprehensive Treatment" (F. Franks, Ed.), The physics and physical chemistry of water, Vol. 1, pp. 189–199. Plenum Press, New York.

Heimovaara, T. J., Bouten, W., and Verstraten, J. M. (1994). Frequency-domain analysis of time-domain reflectometry wave forms. 2. A 4-component complex dielectric mixing model for soils. *Water Resour. Res.* **30**, 201–209.

Kelleners, T. J., Soppe, R. W. O., Robinson, D. A., Schaap, M. G., Ayars, J. E., and Skaggs, T. H. (2004). Calibration of capacitance probe sensors using electric circuit theory. *Soil Sci. Soc. Am. J.* **68**, 430–439.

Klute, A. (1986). Water retention: Laboratory methods. *In* "Methods of Soil Analysis, Part 1: Physical and mineralogical methods" (A. Klute, Ed.), 2nd ed., pp. 635–662. ASA, Madison, WI.

Klute, A., and Dirksen, C. (1986). Hydraulic conductivity and diffusivity: Laboratory methods. *In* "Methods of Soil Analysis. Part 1: Physical and mineralogical methods" (A. Klute, Ed.), 2nd ed., pp. 687–734. ASA, Madison, WI.

Kuraz, V. (1982). Testing of a field dielectric soil moisture meter. ASTM Geotech. Test. J. 4.

Ledieu, J., Deridder, P., Declerck, P., and Dautrebande, S. (1986). A method of measuring soil moisture by Time-Domain Reflectometry. *J. Hydrol.* **88**, 319–328.

Malano, H. M., Turral, H. N., and Wood, M. L. (1996). Surface irrigation management in real time in southeastern Australia: Irrigation scheduling and field application. ''Irrigation Scheduling: From Theory to Practice—Proceedings of the ICID/FAO Workshop.'' FAO, Rome, Italy.

Mead, R. M., Ayars, J. E., and Liu, J. (1995). Evaluation of the Sentek EnviroSCAN RT5 capacitance probe: Laboratory calibration and field analysis. A report submitted by the Water Management Research Laboratory ARS-USDA. Fresno, CA.

Moons, B., and Gilmore, L. (2001). Irrigation scheduling using C-Probes. ''Proceedings of the second annual Manitoba Agronomists Conference,'' pp. 204–206. University of Manitoba, Winnipeg, MB.

Morgan, K. T., Parsons, L. R., Wheaton, T. A., Pitts, D. J., and Obreza, T. A. (1999). Field calibration of a capacitance water content probe in fine sand soils. *Soil Sci. Soc. Am. J.* **63**, 987–989.

Morgan, K. T., Parsons, L. R., and Wheaton, T. A. (2001). Comparison of laboratory- and field-derived soil water retention curves for a fine sand soil using tensiometric, resistance and capacitance methods. *Plant Soil* **234**, 153–157.

Muldon, D., Hickey, M., and Hoogers, R. (1999). Water use by furrow-irrigated onions on a clay soil. Farmer's Newsletter No. 183. Horticulture, Irrigation Research and Extension Committee. Institute of Sustainable and Irrigated Agriculture, Tatura, Victoria.

Nachabe, M., Masek, C., and Obeysekera, J. (2004). Observations and modeling of profile soil water storage above a shallow water table. *Soil Sci. Soc. Am. J.* **68**, 719–724.

Nachabe, M., Shah, N., Ross, M., and Vomacka, J. (2005). Evapotranspiration of two vegetation covers in a shallow water table environment. *Soil Sci. Soc. Am. J.* **69**, 492–499.

Noborio, K. (2001). Measurement of soil water content and electrical conductivity by time domain reflectometry: A review. *Comput. Electron. Agr.* **31**, 213–237.

Obreza, T. A., and Pitts, D. J. (2002). Effective rainfall in poorly drained microirrigated citrus orchards. *Soil Sci. Soc. Am. J.* **66**, 212–221.

Olden, S., Buss, P., Fares, A., and Dalton, M. (2002). Adopting best management practices for winery wastewater re-use onto a woodlot Irrigation Association Annual Meeting, LA.

Or, D., and Wraith, J. M. (1999). Temperature effects on soil bulk dielectric permittivity measured by time domain reflectometry: A physical model. *Water Resour. Res.* **35**, 371–383.

Paltineanu, I. C., and Starr, J. L. (1997). Real-time soil water dynamics using multisensor capacitance probes: Laboratory calibration. *Soil Sci. Soc. Am. J.* **61**, 1576–1585.

Paltineanu, I. C., and Starr, J. L. (2000). Preferential water flow through corn canopy and soil water dynamics across rows. *Soil Sci. Soc. Am. J.* **64**, 44–54.

Parlange, M. B., Cahill, A. T., Nielsen, D. R., Hopmans, J. W., and Wendroth, O. (1998). Review of heat and water movement in field soils. *Soil Till. Res.* **47**, 5–10.

Pepin, S., Livingston, N. J., and Hook, W. R. (1995). Temperature dependent measurement errors in time-domain reflectometry determinations of soil water. *Soil Sci. Soc. Am. J.* **59**, 38–43.

Persson, M. (1997). Soil solution electrical conductivity measurements under transient conditions using time domain reflectometry. *Soil Sci. Soc. Am. J.* **61**, 997–1003.

Persson, M., and Berndtsson, R. (1998). Texture and electrical conductivity effects on temperature dependency in time domain reflectometry. *Soil Sci. Soc. Am. J.* **62**, 887–893.

Pleban, S., and Israeli, I. (1989). Improved approach to irrigation scheduling programs. *J. Irrig. Drain. E.-ASCE* **115**, 577–587.

Polyakov, V. O., Fares, A., and Ryder, M. H. (2005). Calibration of a capacitance system for measuring water content of tropical soil. *Vadoze Zone J.* **4**, 1004–1010.

Robinson, D. (1999). A comparison of soil-water distribution under ridge and bed cultivated potatoes. *Agr. Water Manag.* **42**, 189–204.

Robinson, D. A. (2004). Calculation of the dielectric properties of temperate and tropical soil minerals from ion polarizabilities using the Clausius-Mosotti equation. *Soil Sci. Soc. Am. J.* **68**, 1780–1785.

Sentek, D. A. (2003). "EasyAG Installation Guide." Sentek Sensor Technologies, Stepney, Australia.

Seyfried, M. S., and Murdock, M. D. (2001). Response of a new soil water sensor to variable soil, water content, and temperature. *Soil Sci. Soc. Am. J.* **65**, 28–34.

Smajstrla, A. G., and Harrison, D. S. (1982). Irrigation scheduling. Agricultural Engineering Extension Report 82-2. IFAS, University of Florida, Gainesville.

Sodek, F., Carlisle, V. W., Collins, L. C., Hammond, L. C., and Harris, W. G. (1990). "Characterization Data for Selected Florida Soils." Soil Characterization Lab. University of Florida, Gainesville, FL.

Starr, J. L., and Paltineanu, I. C. (1998). Soil water dynamics using multisensor capacitance probes in non-traffic interrows of corn. *Soil Sci. Soc. Am. J.* **62**, 114–122.

Starr, J. L., and Paltineanu, I. C. (2002). Capacitance devices. *In* "Methods of Soil Analysis. Part 4. Book Ser. 5" (J. H. Dane and G. C. Topp, Eds.). SSSA, Madison, WI.

Starr, J. L., and Timlin, D. J. (2004). Using high resolution soil moisture data to assess soil water dynamics in the vandose zone. *Vandoze Zone J.* **3**, 926–935.

Thompson, R. B., Gallardo, M., and Fernandez, M. D. (2004). Irrigation scheduling of drip-irrigated vegetable crops grown in greenhouses using continuous soil moisture monitoring. *In* "ISHS Acta Horticulturae IV" (R. L. Snyder, Ed.), p. 664. International Symposium on Irrigation of Horticultural Crops, Davis, CA.

Topp, G. C., and Ferre, T. (2002). Gravimetric using microwave oven-drying. *In* "Methods of Soil Analysis" (J. H. Dane and G. C. Topp, Eds.), pp. 425–446. Part 4. Book Ser. 5. SSSA, Madison, WI.

Topp, G. C., Davis, J. L., and Annan, A. P. (1980). Electromagnetic determination of soil-water content–measurements in coaxial transmission lines. *Water Resour. Res.* **16**, 574–582.

Tyndale-Biscoe, P., and Malano, H. M. (1995). A laboratory comparison of some currently available soil moisture monitoring devices. *Agr. Eng. Aus.* **1**, 18–24.

van Genuchten, M. T. (1980). A closed-form equation for predicting the hydraulic conductivity of unsaturated soil. *Soil Sci. Soc. Am. J.* **44**, 892–898.

van Genuchten, M. T., Leij, F. J., and Yates, R. S. (1991). The RETC code for quantifying functions of unsaturated soils. EPA/600/2-91/065.

Wang, J. R. (1980). The dielectric properties of soil-water mixtures at microwave frequencies. *Radio Sci.* **15**, 977–985.

Weast, R. C. (Ed.) (1986). "Handbook of Chemistry and Physics." CRC Press, Boca Raton, FL.

White, I., Knight, J. H., Zegelin, S. J., and Topp, G. C. (1994). Considerations on the use of time-domain reflectometry (TDR) for measuring soil-water content—comment. *Eur. J. Soil Sci.* **45**, 503–508.

Wraith, J. M., and Or, D. (1999). Temperature effects on soil bulk dielectric permittivity measured by time domain reflectometry: Experimental evidence and hypothesis development. *Water Resour. Res.* **35**, 361–369.

Wu, K. G. (1998). Measurement of soil moisture change in spatially heterogeneous weathered soils using a capacitance probe. *Hydrol. Process.* **12**, 135–146.

Yang, H., Reichert, P., Abbaspour, K. C., and Zehnder, A. J. B. (2003). A water resources threshold and its implications for food security. *Environ. Sci. Technol.* **37**, 3048–3054.

Yu, C., Warrick, A. W., and Conklin, M. H. (1999). Derived functions of time domain reflectometry for soil moisture measurement. *Water Resour. Res.* **35**, 1789–1796.

This page intentionally left blank

Synchrotron Radiation Infrared Spectromicroscopy: A Noninvasive Chemical Probe for Monitoring Biogeochemical Processes

H.-Y. N. Holman[1,2] and M. C. Martin[3]

[1]Ecology Department, Earth Sciences Division,
Lawrence Berkeley National Laboratory, University of California,
Berkeley, California 94720
[2]Virtual Institute for Microbial Stress and Survival,
Lawrence Berkeley National Laboratory, University of California,
Berkeley, California 94720
[3]Advanced Light Source Division,
Lawrence Berkeley National Laboratory, University of California,
Berkeley, California 94720

A long-standing desire in biogeochemistry is to be able to examine the cycling of elements by microorganisms, as the processes are happening on surfaces of earth and environmental materials. Over the past decade, physics, engineering, and instrumentation innovations have led to the introduction of synchrotron radiation-based infrared (IR) spectromicroscopy. Spatial resolutions of less than 10 micrometers (μm) and photon energies of less than an electron volt make synchrotron IR spectromicroscopy noninvasive and useful for following the course of biogeochemical processes on complex heterogeneous surfaces of earth and environmental materials. In this chapter, we will first briefly describe the technology and then present

79

several examples demonstrating its application potentials in probing and imaging biogeochemical processes.   © 2006, Elsevier Inc.

I. INTRODUCTION

Microorganisms are important agents in the geochemical cycling of elements. For example, they can change the speciation of metal ions and organic carbons in soils and sediments by releasing complexing agents and by enzymatically catalyzing reactions (Barker and Banfield, 1996, 1998; Benzerara et al., 2005; Cooper et al., 2005; Edwards et al., 2000, 2001; Ehrlich, 1998, 2000; Emerson and Ghiorse, 1993; Ghiorse and Hirsch, 1979; Jones et al., 2003; Kalinowski et al., 2000; Lajtha and Schlesinger, 1988; Lovley and Woodward, 1996; Miller et al., 2004; Tebo et al., 1997). They can also modify the composition of pore fluid and groundwater through controlled mineral weathering and precipitation (Andrejchuk and Klimchouk, 2001; Barker and Banfield, 1998; Bennett et al., 2000; Benzerara et al., 2002, 2005; Cacchio et al., 2004; Cheah et al., 2003; Edwards et al., 2005; Ehrlich, 1994, 1998; Engel et al., 2004; Goodhue et al., 2005; Joeckel and Clement, 2005; Maurice et al., 2001; Mcmahon and Chapelle, 1991; Renault et al., 1998; Sanchez-Moral et al., 2003; Spilde et al., 2005; Welch et al., 2002). Most importantly, they can transform many environmental pollutants to less toxic species (Aksu, 2005; Francis et al., 2000, 2004; Lack et al., 2002; Lovley and Phillips, 1992; Lovley et al., 1993a,b; Merroun et al., 2005; Neal et al., 2004a; Osborne and Ehrlich, 1976; Panak et al., 2002; Phillips et al., 1995; Suzuki and Banfield, 2004; Watson and Ellwood, 2003; Watson et al., 2000; Zouboulis and Katsoyiannis, 2005). With the discovery of diverse microbial communities thriving in every possible environment (Amend, 2004; Baker and Banfield, 2003; Balkwill and Ghiorse, 1985; Burton and Lappin-Scott, 2005; Campen et al., 2003; Dees and Ghiorse, 2001; Douglas and Douglas, 2001; Edwards et al., 2003; Fredrickson et al., 2004; Ghiorse and Chapnick, 1983; Krumholz et al., 1997; Leveille et al., 2000; Macalady and Banfield, 2003; Pennisi, 2000; Schabereiter-Gurtner et al., 2002; Sinclair and Ghiorse, 1989; Templeton et al., 2005; Wellsbury et al., 2002; Zhang and Lanoil, 2004), researchers in biogeochemistry are now increasingly focused on expanding their understanding of roles of environmental microorganisms at a more fundamental level. Many important microbial processes happen at the interface between microorganisms and earth or environmental materials. This necessitates a more comprehensive study and analysis of how microorganisms through their wide range of metabolic capabilities interact with their environments, especially at

surfaces of earth and environmental materials. This surface biogeochemistry can be highly variable at a microscopic level because of the small-scale (ranging from one micron to hundreds of microns) surface heterogeneity, which involves the distributions of clusters of mineral-inhabiting microorganisms and reactive molecules of metal oxides and organic molecules. The methodology commonly employed to study this type of heterogeneous biogeochemical phenomenon is a combination of microscopic imaging and synchrotron radiation (SR)-based X-ray spectroscopy techniques. The interested readers can read reviews (Brown and Parks, 2001; Gordon and Sturchio, 2002) and other relevant studies (Amonette *et al.*, 2003; Arnesano *et al.*, 2003; Benison *et al.*, 2004; Benzerara *et al.*, 2005; Cooper *et al.*, 2005; De Stasio *et al.*, 2001; Fein *et al.*, 2002; Foriel *et al.*, 2004; Francis *et al.*, 2004; Jones *et al.*, 2003; Jurgensen *et al.*, 2004; Khijniak *et al.*, 2005; Lack *et al.*, 2002; Li *et al.*, 2003; Lieberman *et al.*, 2003; Neal *et al.*, 2004a,b; Nesterova *et al.*, 2003; Panak *et al.*, 2002; Pickering *et al.*, 2001; Prange *et al.*, 2002a,b; Renshaw *et al.*, 2005; Saita and Maenosono, 2005; Sarret *et al.*, 2005; Suzuki *et al.*, 2003; Tebo *et al.*, 2004, 2005; Templeton *et al.*, 2005; Toner *et al.*, 2005; Twining *et al.*, 2004; Vogt *et al.*, 2003; Watson and Ellwood, 2003; Wildung *et al.*, 2004; Zouboulis and Katsoyiannis, 2005). SR-based X-ray spectromicroscopy studies have provided important and unique information about how microorganisms interact with earth and environmental materials. However, the energy range associated with SR-based X-ray spectromicroscopy techniques is between tens and thousands of electron volts (eV), which can adversely affect, harm, or even kill the microorganisms. Consequently, it has limited the use of these techniques to measuring the biogeochemical actions only at single time points.

Being able to measure real-time sequential molecular changes in a biogeochemical system, as they are happening on surfaces of earth and environmental surfaces, has been a long-standing scientific desire in biogeochemistry. The new availability of SR-based infrared (IR) sources to the scientific community in the 1990s provided this opportunity. Our group began developing an SR-based Fourier transform infrared (SR-FTIR) spectromicroscopy approach in 1998 for studying biogeochemical transformation of environmental pollutants, choosing the reduction of hexavalent chromium by living microorganisms on mineral surfaces as the initial application (Holman *et al.*, 1999). Prior to the availability of SR-based IR facilities, these type of *in vivo* and *in situ* measurements were very difficult for two reasons. First, earth materials inherently have low IR reflectivity surfaces. High-quality IR spectroscopy measurements of earth and environmental materials require a high-IR photon flux on small surface areas. Without an SR-based source, one often needs to coadd thousands to tens of thousands of spectral scans, which can be prohibitively time consuming. Second, the IR measurements of live microorganisms had been problematic.

Investigators were required to feed bacteria with a substantial quantity of deuterated substrates in order to obtain sufficient signal-to-noise spectra (Cameron *et al.*, 1983). However, deuterated substrates are known to alter activities and even produce stresses in microorganisms (Newo *et al.*, 2004; Pshenichnikova *et al.*, 2004).

There are 13 synchrotron IR spectromicroscopy facilities around the world with several more under construction or planned (see, for instance, http://www.lightsource.ca; http://www.diamond.ac.uk). Within the United States, there are four active synchrotron IR facilities with microscopy capabilities: (1) the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory), (2) the Synchrotron Radiation Center (SRC, University of Wisconsin-Madison), (3) the Center for Advanced Microstructures and Devices (CAMD, Louisiana State University), and (4) the Advanced Light Source (ALS, Lawrence Berkeley National Laboratory); each has similar capabilities and uniqueness. All four are user facilities (i.e., available to qualified scientists). The first SR-FTIR spectromicroscopy experiments relevant to earth materials were the measurements of composition of clay mineral surfaces (Bantignies *et al.*, 1995), followed shortly by measurements of hydrous minerals (Lu *et al.*, 1999) and of entrapped oil–water inclusions (Guilhaumou *et al.*, 1998). The first SR-FTIR spectromicroscopy experiment relevant to cells, although not performed on bacteria, was chemical imaging of single human cells (Jamin *et al.*, 1998), bones (Miller *et al.*, 1998), and plant tissues (Wetzel *et al.*, 1998). The first SR-FTIR spectromicroscopy experiments relevant to biogeochemistry in vadose environments were the *in situ* and *in vivo* sequential measurements of reduction of hexavant chromium by a colony of basalt-inhabiting bacteria (Holman *et al.*, 1999) and of metabolization of pyrene by a colony of soil-inhabiting bacteria from a Superfund site (Holman *et al.*, 2002b). The first SR-FTIR spectromicroscopy experiments of aqueous environments was the characterization of metal–cyanobacteria sorption reactions (Yee *et al.*, 2004b).

The purposes of this chapter are to familiarize readers with SR-FTIR spectromicroscopy and to realize key issues requiring consideration prior to its application to biogeochemistry. Rather than presenting a comprehensive review of all applications of SR-FTIR spectromicroscopy, we shall focus on contents that illustrate the requirements and utility of SR-FTIR spectromicroscopy as a noninvasive molecular probe for tracking molecular changes in a biogeochemical system. The interested readers should read review articles on applications of SR-FTIR spectromicroscopy to other related areas, including ecological and agricultural sciences (Raab and Vogel, 2004), surface and environmental sciences (Hirschmugl, 2002a,b), and biology and biomedicine (Dumas *et al.*, 2004; Holman *et al.*, 2000a,b; Miller *et al.*, 2000, 2002; Wetzel *et al.*, 2005). Readers can also find

information in the reports on the applications of SR-FTIR spectromicroscopy, characterizing chemistry of fossil microorganisms (Foriel *et al.*, 2004), susceptibility of plants to mildew (Vogel *et al.*, 2002, 2004), structural–chemical features of feeds and plants (Yu, 2005a,b; Yu *et al.*, 2003, 2004), transport of pollutants in plants (Dokken *et al.*, 2005a,b,c), carbon in interplanetary dust particles (Bradley *et al.*, 2005), and microbial mineralization and silicification processes (Benning *et al.*, 2002, 2003, 2004a,b; Yee and Benning, 2002; Yee *et al.*, 2003, 2004a,b).

II.   SR-FTIR SPECTROMICROSCOPY

SR-FTIR spectromicroscopy takes advantage of three existing technologies: (1) the well-known sensitivity and noninvasive nature of mid-IR spectroscopy to chemical functional groups in molecules and their conformations, (2) the convenience of a microscope to locate areas for molecular and composition analysis, and (3) the high signal-to-noise ratio provided by a noninvasive SR-based IR light source. Mid-IR spectroscopy is also a rapid, reagentless, and nondestructive analytical technique, which has a wide range of applications in biosciences, molecular or organismal. In the later section, we shall describe SR-FTIR spectromicroscopy and its issues as a biogeochemical microprobe following the background section.

A.   Background

The application of SR-based IR light as a source of energy to study biogeochemical processes is an experimental effort. It is based on the principle of vibrational spectroscopy of molecules in the IR region. FTIR spectroscopy of a sample is the use of a Fourier transform interferometer to study the interaction of incoming IR light with molecules in the sample. The instrumentation for Fourier transform spectrometry includes a source of IR light, a means to measure each photon energy, an interface allowing this discreet light to be transmitted or reflected by the sample, a detector, and a data recording and analysis system. The typical measurement recorded is a spectrum of IR absorbance in the sample as a function of the wavelength of IR light (typically expressed in units of wavenumber, cm^{-1}). Atoms of a molecule vibrate with characteristic frequencies (normal modes) governed by their chemical bonds and symmetry environment. Incoming IR light will be absorbed by the molecule, if the following two criteria are met: (1) the frequency of the IR light matches exactly the frequency of the vibrational mode, and (2) the vibration causes an asymmetric change in

the charge distribution within the molecule (dipole moment). The strength of the dipole moment correlates with the strength of the absorption. IR spectroscopy is, therefore, sensitive to the presence of many chemical functional groups (structural fragments) in molecules in samples, and taken together, the set of vibration modes are unique for every molecular configuration. (More in-depth readings regarding vibrational spectroscopy of molecules and macromolecules can be found at the web site: http://infrared.als.lbl.gov/FTIRinfo.html).

IR radiation was discovered by William Herschel in 1800 during his investigations of the solar spectrum. However, the potential of using IR light energy as a source for spectroscopy was not realized until the later part of the nineteenth century. W. dew. Abney and E. R. Festing were the first researchers to successfully use IR radiation as a light source to obtain IR spectra of almost 50 organic compounds and recommended the use of IR spectroscopy as an analytical tool [Phil. Trans. Roy. Soc. London (1882). **172**, 887–918]. In 1905, William Weber Coblentz referenced this empirical evidence and demonstrated in his investigations of IR spectra that different atomic and molecular groupings absorbed specific and characteristic wavelengths. However, it is the application of Fourier transform spectroscopy in conjunction with a Michelson interferometer in 1911 by Rubens and Woods that laid the foundation of modern FTIR spectroscopy. Difficulties associated with computing Fourier transformations manually had hindered the application of the technology. Throughout the first half of the twentieth century, its applications were limited mostly to researchers in physics and astronomy, although it had found its place in the Second World War as a useful diagnostic tool in determining the concentration and purity of butadiene in synthetic rubber.

In 1949, Barer *et al.* (1949), Gore (1949), and Blout *et al.* (1949) demonstrated the potential importance of joining IR spectroscopy with microscopy to seeing microscopic structures in a sample, analyzing molecular chemistry, and relating composition with the observed microstructures (Barer, 1949, 1953, 1954; Barer and Joseph, 1954; Bird and Blout, 1952; Blout, 1953). With continued improvement in high-quality detectors and spectrometers and the rapid development in microprocessor technology, the first practical IR microspectrometer, which was conceived by Coates *et al.* (1953), became available commercially in 1978. Shortly afterwards, the innovative application of the Fast Fourier transform algorithm (Cooley and Tukey, 1965) to FTIR spectroscopy, aided by the availability of low-cost high-speed computers, has led to an explosive growth in mid-IR spectromicroscopy instrumentation primarily in the 1990s, and making it a popular analytical approach to detecting, identifying, and quantifying many molecular species mostly in biological samples.

The IR sources used in these FTIR spectromicroscopy (or microspectro-scopy) instruments are thermal emission elements (or thermal globars) that produce a graybody spectrum from a filament heated to between 1000 and 2000 K. These globars can be rod-, coil-, or u-shaped, physically moderate in size (at least several millimeters), and typically radiate in all directions. As shown in Fig. 1, the FTIR bench optics collect the light, then collimate and pass it through the scanning interferometer. Next, this modulated light is directed into an IR microscope. The IR microscope objective and condenser optics are reflective and focus the IR light to a small spot on a sample. Finally, the light that the sample reflects or transmits is collected, focused onto a detector, and processed by a computer to produce an IR spectrum. The first FTIR spectromicrosocpy experiments were measurements on coals (Brenner, 1983) and polymers (Peitscher, 1986) during the first half of the 1980s. During the early 1990s, the first set of experiments performed on biological materials were on isolated human cells (Daoud *et al.*, 1988), tissue specimens (Centeno and Specht, 1992; Centeno *et al.*, 1992), and plant cells (Mccann *et al.*, 1992). The FTIR spectromicroscopy measurements of
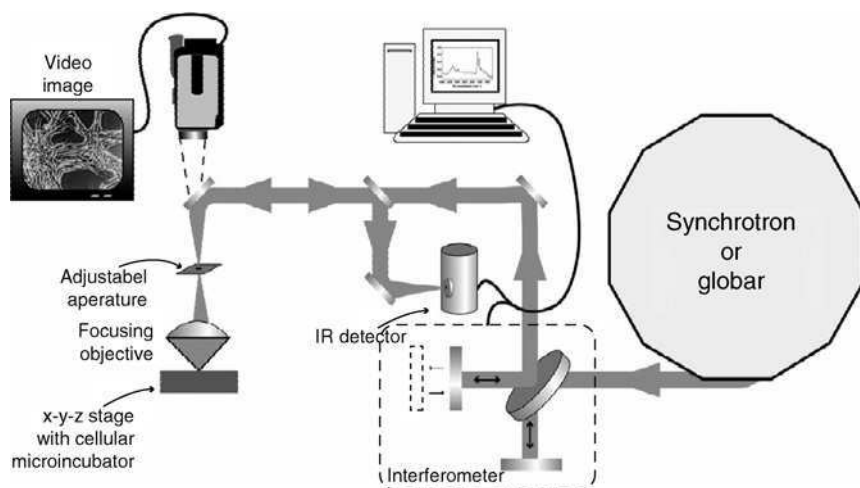

Figure 1 Schematic diagram of Fourier transform infrared (FTIR) spectromicroscopy experimental setup. Mid-IR radiation from either a synchrotron or a globar is transported to an FTIR interferometer bench. After modulation by the interferometer, an IR microscope with all-reflecting optics focuses the beam onto the sample. Microbial or biogeochemical samples can be placed inside an on-stage mini-incubator with environmental controls. The stage is computer controlled and rasters the sample in the x-y-z plane to ±0.1 μm precision to obtain spectral maps across the sample. The light reflected from the sample is collected by the same microscope optics and sent to an IR detector. A computer performs a Fourier transform on the measured interferogram to obtain an IR spectrum. (Reproduced with permission from *Spectrosc.-Int. J.*, 2003, **17**, 139–159. IOS Press.)

bacteria were first conducted in conjunction with chemometrics to discriminate different bacterial strains (Kansiz *et al.*, 1999; Lang and Sang, 1998) almost 10 years later. The popularity of FTIR spectromicroscopy in research (as measured in terms of numbers of publications involving the applications of FTIR spectromicroscopy) soared (Fig. 2).

However, light emitted from thermal globars does not provide sufficient signal-to-noise for the detailed spectral interpretation of microbial assemblies of several to tens of microns. Such measurements were especially difficult to obtain if the microorganisms were on surfaces of earth materials with low IR reflectivities. High-quality IR spectroscopy measurements of these materials require high-IR photon flux focused to a small spot (brightness). The brightness attainable in IR spectromicroscopy is governed primarily by how point-like the source is. Thermal emission sources, for example, can be focused with an IR microscope to a spot with a 75–100-μm diameter. To measure something smaller, such as a small microbial colony on a mineral surface, one needs to use an aperture to mask away part of the incoming light, or distribute the incoming light among an array of detectors. The use of an aperture can significantly reduce the signal strength.

Our earlier work showed that the brightness (flux per unit area) attainable from a conventional thermal globar IR source is not sufficient for the use of FTIR spectromicroscopy to study biogeochemical processes on mineral surfaces without a surface treatment (Holman *et al.*, 1998). According to
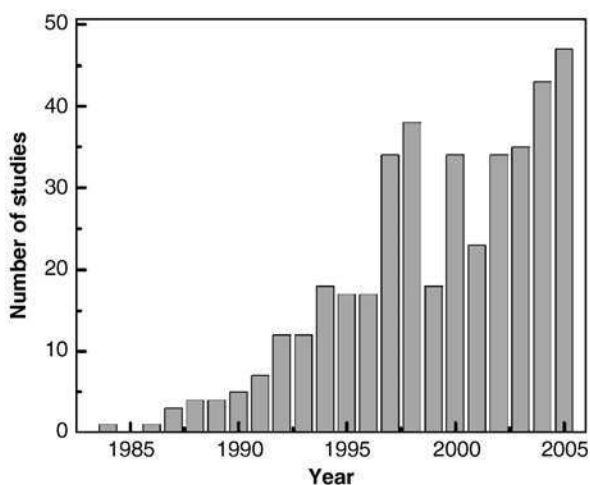

Figure 2   An almost fivefold increase in the popularity of FTIR spectromicroscopy (or microspectroscopy) as indicated by the number of published articles that present FTIR spectromicroscopy measurements.

the earlier discussion, it follows that one needs an IR source that acts like a true point source, that is, a source that could be focused to a diffraction-limited spot size to optimize for maximum brightness. With the f/1 optics (i.e., the primary focal ratio is $f/1$), this yields a diffraction-limited spatial resolution of approximately the wavelength of the light without loosing any signal strength. This is the benefit of using a synchrotron as an IR source.

B. SYNCHROTRON IR LIGHT SOURCES

A synchrotron is a high-energy electron storage ring, optimized for the production and collection of the intense light radiated by the electrons upon acceleration. In modern synchrotrons, electrons are first accelerated to near the speed of light and then injected into the storage ring (Fig. 3A). Electrons that travel near the speed of light are called relativistic electrons. The storage ring is designed to make the traveling electrons complete a loop via a series of bending magnets and straight sections. When the electrons encounter a magnetic field, they are deflected and emit electro-magnetic radiation—light. Typical bending magnets have a magnetic field strength of ≈ 1 T. This field
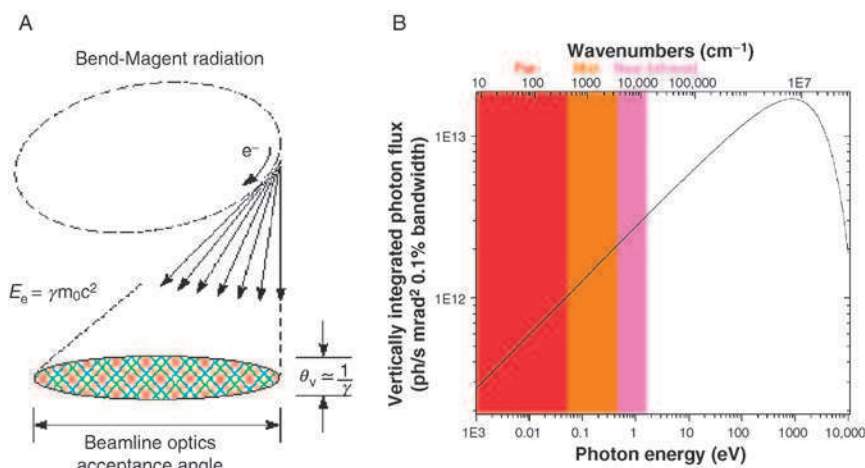

Figure 3 An overview of synchrotron radiation. (A) Guided by a series of bending magnets and straight sections, relativistic electrons inside a storage ring complete a loop. When the relativistic electrons encounter a magnetic field, they are deflected and they emit electromagnetic radiation with energy photons up to hard X-rays. (B) This so-called bending magnet spectrum extends from very low energies (far-IR) continuously to a critical energy in the soft or hard X-ray, depending on the energy of the synchrotron. The radiation pattern from relativistic electrons is such that its effective source size can be considered very close to an ideal point source.

strength, coupled with the velocity of the electrons, determines the energies of the emitted photons. This means that higher velocities (higher energy storage rings) and/or higher magnetic fields produce higher energy photons up to hard X-rays. This so-called bending magnet spectrum (Fig. 3B) extends from very low energies (far-IR) continuously to a critical energy in the soft or hard X-ray, depending on the energy of the synchrotron. Because the radiation pattern from relativistic electrons is such that the opening angle of the emitted radiation is very small, the effective source size of the IR radiation source is dominated by diffraction, and thus can be considered as very close to an ideal point source. Interested readers are directed to an informative overview of SR by Sham and Rivers (Sham and Rivers, 2002).

As expected, in the mid-IR region — 400–4000 cm^{-1} — the effective source size for a typical synchrotron light source is dominated by diffraction (Carr *et al.*, 1995; Holman *et al.*, 2003; Reffner *et al.*, 1995, 1997). This means that for SR-FTIR spectromicroscopy the IR beam is focused visibly to a spot with a diameter of about 0.7 times the wavelength, which for the mid-IR wavelengths of 2.5–25 μm yields a spatial resolution of 1.7–17 μm (Levinson *et al.*, 2006) This is smaller than a typical microbial colony, thus, providing a spatial resolution smaller than a microbial colony with hundreds to a thousand times the brightness of conventional IR sources (Fig. 4).
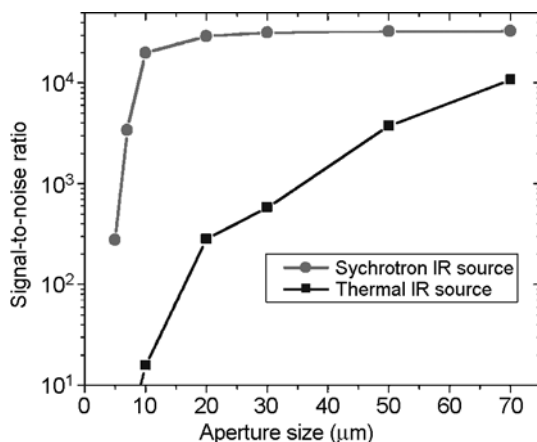

Figure 4 Comparison of measured noise around 100% reflectance for the thermal and synchrotron IR sources with different aperture size expressed in terms of signal-to-noise ratio on a log scale as a function of aperture diameters for the synchrotron and thermal IR sources. The synchrotron source extends FTIR spectromicroscopy to below 20 μm spatial resolution with a signal-to-noise advantage over conventional IR sources of at least 100. (Reproduced with permission from *Spectrosc.-Int. J.*, 2003, **17**, 139–159. IOS Press.)

To demonstrate the advantage of using a synchrotron as an IR energy source for FTIR spectromicroscopy, we describe here three studies that compare the measured signal-to-noise ratio as a function of aperture size for a conventional thermal globar IR source and the synchrotron. The first two studies were performed using a Thermo Nicolet Nexus 870 FTIR bench and a Thermo SpectraTech Contiuum IR microscope at the ALS beamline 1.4.3. The third study was performed at LURE (Laboratoire pour l'Utilisation du Rayonnement Electromagnétique, Orsay, France).

During the first experiment, we measured 100% reflection lines utilizing a gold-coated glass sample for both sources and for various aperture sizes. We used an MCT-B detector, coadded 128 scans for background and sample measurements at a spectral resolution of 4 cm^{-1} and a scanning mirror velocity of 1.8988 cm s^{-1}. The signal-to-noise value centered at 2500 cm^{-1} was obtained for both the conventional thermal source and the synchrotron source, using different aperture settings. The value was calculated by dividing the measured single beam intensity at this wavenumber by the corresponding root-mean-square (RMS) noise value. The advantage of signal-to-noise improvement is shown in Fig. 4. For the thermal globar source, the signal-to-noise level decreases significantly as the aperture diameter decreases. Signals become essentially unusable at aperture sizes below 20×20 μm^2. This is because the size of the thermal globar source, when focused to a surface, is greater than 70×70 μm^2 (Carr, 1999; Holman *et al.*, 2003; Reffner *et al.*, 1995, 1997). By reducing the aperture size, one simply reduces the total IR signal. For the synchrotron source, the signal-to-noise ratio is significantly better for almost all aperture sizes, although the ratio also begins to decrease when the aperture size is smaller than the diffraction-limited spot size. This difference is because of the focused spot size of the synchrotron source, which is diffraction limited (1.7–17 μm in diameter) (Carr, 2001; Levinson *et al.*, 2006). Consequently, its signal-to-noise ratio is only affected when the aperture size becomes less than the diffraction-limited spot size (starting with the longest wavelengths within the mid-IR region).

The second experiment compares the signal-to-noise ratio on earth materials. In Fig. 5, there is a geological example of how the high-brightness (i.e., high signal-to-noise ratio) of the synchrotron IR source makes very time consuming and difficult measurements possible. A tiny piece of ocean basalt was mounted in a diamond anvil cell to achieve extremely high pressures and the IR absorbance of the sample was measured at a pressure of 32 GPa. When using a conventional FTIR spectromicroscopy system, a 7-h signal averaging of over 60,000 scans was required to begin to detect the spectral features. With a synchrotron source, a significantly improved signal-to-noise was achieved after only 2 min of averaging 256 scans (Panero *et al.*, 2003).
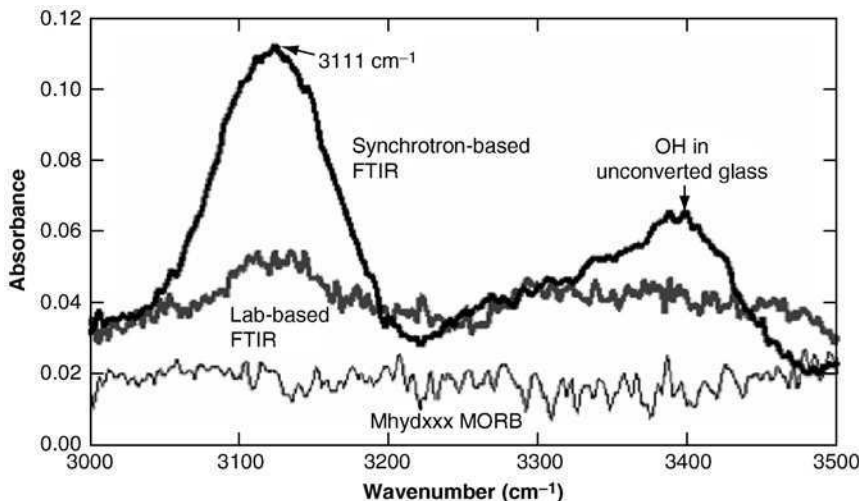
Figure 5   Spectra of a sample synthesized at 32 (±2) GPa and 2850 (±150) K, comparing results from a synchrotron-based system [black line, advanced light source (ALS) beamline 1.4.3; Nicolet Magna 760 with KBr beamsplitter and an MCT detector] to the spectrum from a lab-based system (gray line, Bruker IFS-66v using a CaF2 beamsplitter and an InSb detector). The collection time for the synchrotron-based system was about 2 min (256 scans, top) compared to about 7 h (60,000 scans, bottom) for the lab-based system. While both show a distinct peak at 3111 cm^{-1} corresponding to OH vibrations in stishovite, the synchrotron-based spectrum has a better signal-to-noise ratio, as well as better spatial resolution. There is no detectable absorption at 3450 cm^{-1}, where OH in Mg-perovskite is expected to absorb. A control experiment was performed on a dry, synthetic basalt glass starting material (sample 1114b_6); synthesis conditions were 33 (±1) GPa and 2130 (±150) K. No absorption features were found in the 3000–3500 cm^{-1} region for this sample (thin line, bottom), again collected by synchrotron FTIR (Panero *et al.*, 2003). (Reproduced with permission from *J. Geophys. Res.-Sol. Ea*, 2003, **108**, 2039–2047, American Geophysical Union.)

The third experiment compared the signal-to-noise ratio on biological materials. In Fig. 6, there are FTIR spectra from a single living cell using a $6 \times 6 \ \mu m^2$ aperture (Figure courtesy of P. Dumas). In this experiment, the investigators clearly demonstrate that even with significantly longer averaging times, the signal-to-noise of the globar measurement is so poor that the data are not usable, whereas the synchrotron-based measurements show all the fine spectral structures required for detailed analysis (Dumas and Miller, 2003).

C.   Synchrotron IR Spectromicroscopy of Biogeochemical Systems

The experimental evidence described earlier reveals that for studying a surface phenomenon with a spatial resolution ranging from 1.7 to 17 μm,
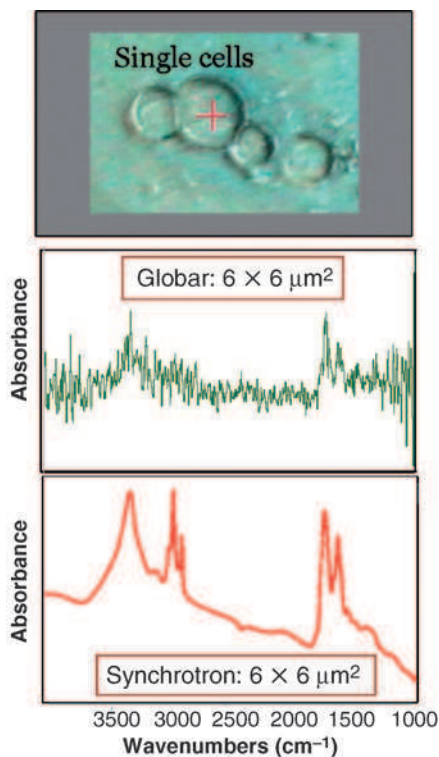
Figure 6 FTIR spectra of a single cell using a 6×6 μm^2 aperture, comparing results from a synchrotron-based system (red, at Laboratoire pour l'Utilisation du Rayonnement Electromagnétique, Orsay, France) to the spectrum from a lab-based system (green). The collection time for the synchrotron-based system was about 16 s (32 scans, bottom) compared to about 500 s (1000 scans, middle) for the lab-based system. These investigators clearly demonstrate that even with significantly longer averaging times, the signal-to-noise of the globar measurement is so poor that the data are not usable, whereas the synchrotron-based measurements show all the fine spectral structures required for detailed analysis. (Figure courtesy of P. Dumas.)

the signal-to-noise ratio provided by a synchrotron IR source is up to 1000 times better than the signal-to-noise ratio provided by a thermal source. Since the SR-based IR beam does not induce any detectable side-effects in live cells (Holman *et al.*, 2002a) and has negligible sample heating effect (Martin *et al.*, 2001), SR-FTIR spectromicroscopy is clearly an ideal microprobe for a noninvasive study of heterogeneous biogeochemical processes *in vivo* and *in situ*, for example, individual microbial colonies or larger biological systems in which local biochemistry may have significant spatial variations.

However, because of the complicated nature of biogeochemical systems, one must consider the following issues carefully before applying this technology to probe the successive biogeochemical processes. First, microorganisms are exceedingly sensitive to their immediate environments. To reliably study molecular changes in a chain of biogeochemical events, SR-FTIR spectromicroscopy measurements must be made in well-controlled experiments that simulate their viability and functionality under *in situ* conditions. This is especially important since microbial cells alter earth and environmental materials mostly via their metabolic activities (Ehrlich, 1998, 2000). Such experimental conditions of biogeochemical processes are best conducted under well-controlled conditions that are similar to the *in situ* conditions. Such similarities can at least be at the appropriate temperature, pH, redox potential (Eh), nutrient, chemistry of bulk water, pore water, relative humidity, and gas composition. A good example of the importance of controlling the experimental conditions is temperature effects on microbial transformation of redox sensitive elements such as iron and sulfur. An increase in temperature could increase microbial metabolic activity and oxygen removal (Hines *et al.*, 1982), leading to a decrease in redox potential (Lyons *et al.*, 1979; Sorensen *et al.*, 1979). These changes could cause shifts in the relative importance of specific terminal electron acceptors used in bacterial respiration (Revsbech *et al.*, 1980; Sorensen *et al.*, 1979). The decrease in redox potential can also affect the chemical and physicochemical state of redox-sensitive elements. In addition to changing chemistry both in bacteria and the elements, these variations may also affect the chemistry of the overlying thin film of water through changes in diffusional fluxes and other processes. To reliably study molecular changes in this chain of biogeochemical events, SR-FTIR spectromicroscopy measurements must be made in experiments that simulate *in situ* conditions using well-controlled flow through cells with IR-transparent windows. There are several research groups developing various types of automated microfluidic incubation platforms to provide a controlled mechanism to rapidly manipulate these experimental conditions. Some of these platforms also control the thickness of the water film to allow for the IR observation of the biogeochemical processes in aqueous environments. Others have sensors to provide additional measurements of relevant physiological and geochemical parameters.

Second, a prior knowledge regarding the type of the pollutants and the pathways of their possible biogeochemical transformation is important for the successful application of SR-FTIR spectromicroscopy. For heavy metal and metalloid pollutants, they constitute the most difficult environmental problem because they cannot be destroyed once introduced into the environment. A key goal of using SR-based IR spectromicroscopy is to characterize how intrinsic microorganisms affect the speciation of these heavy metals and metalloids, which dictates the overall mobility, bioavailability,

toxicity, and other health risks in the biosphere. An appropriate SR-FTIR spectromicroscopy experiment is one that allows investigators to obtain such fundamental knowledge as the stability and mobility of the parent metal compounds, their interactions with the microorganisms, and the altered stability and mobility of the intermediate products under *in situ* and *in vivo* conditions. Our approach to this issue has been both fundamental and applied in nature. We often complement the SR-FTIR spectromicroscopy experiments with successive *in vitro* and *in vivo* studies of model systems of varying complexities to approximate membrane permeability, biotransformation, toxicity, and couple them with spectroscopic studies. In doing so, we have been able to identify, at least at a functional group level, the targets to be measured and ensure that these targets are likely to be in the biogeochemical system to be investigated.

A good example is the microbial transformation and detoxification of chromium in earth materials. Chromium is a redox-sensitive metal pollutant that enters the environment primarily from industries such as leather tanning, wood preservation, metal plating, and alloying. The two important oxidation states of chromium commonly found in environments are trivalent [Cr(III)] and hexavalent [Cr(VI)] states, which have widely contrasting mobility and bioavailability. Most Cr(VI) compounds are highly soluble in water and are readily bioavailable to ecological receptors, while most Cr(III) compounds are less water soluble and less bioavailable. Cr(VI) compounds are among the earliest chemicals to be classified as mutagens and human carcinogens (IARC, 1990; Levina *et al.*, 2003; Stern, 1982). Its genotoxic and carcinogenic effects are associated with its ability to enter cells rapidly through nonspecific transport. Intracellular biomolecules, such as polysaccharides, L-ascorbic acid, glutathione, and other reductases, readily reduce Cr(VI) species to form an array of genotoxic Cr(III) complexes and other radicals that can cause single-strand breaks and plasmid DNA nicking, in addition to a wide variety of DNA lesions and additional oxidative damage (Codd and Lay, 2001; Dillon *et al.*, 1997; Levina *et al.*, 1999; Snow, 1991; Sreedhara *et al.*, 1997; Tsou *et al.*, 1997; Voitkun *et al.*, 1998). Biogeochemical factors that can lead to the reduction of Cr(VI) to insoluble and/or nongenotoxic Cr(III) compounds in environments are very significant for reducing chromium toxicity. Many indigenous bacteria in chromium-polluted environments possess a multiplicity of survival mechanisms that can potentially transform soluble chromium to less soluble forms. Our experiments show that some Cr-resistant microorganisms immobilize and reduce Cr(VI) to stable Cr(III)-complexes extracellularly via interactions with diverse groups of biomolecules (Codd and Lay, 1999, 2001; Codd *et al.*, 1997; Gez *et al.*, 2005; Levina *et al.*, 2004) and the formation of genotoxic intermediates Cr(V)- and Cr(IV)-complexes (Kalabegishvili *et al.*, 2003; Tsibakhashvili *et al.*, 2002a). This information, in conjunction

with our earlier SR-FTIR result (Holman *et al.*, 1999), was applied to the design and execution of an additional in-depth SR-FTIR spectromicroscopy study of Cr(VI) transformation on mineral surfaces (Holman, 2004). The speciation of Cr(III) is one of the focal points in the study. There are concerns that Cr(III) [as $Cr(OH)_3$] can be reoxidized to form Cr(VI) compounds (Chinthamreddy and Reddy, 1999). However, our preliminary SR-FTIR spectromicroscopy results indicate that only a small fraction of the Cr(III) compounds is found as $Cr(OH)_3$.

Unlike heavy metals and metalloid pollutants, organic pollutants can be destroyed. Once they have entered into the biosphere, they can be degraded, metabolized, and/or mineralized by many intrinsic bacteria via one of the many possible pathways of different complexities and kinetics (da Silva *et al.*, 2003; Furukawa, 2000, 2003; Furukawa *et al.*, 1993, 2004; Hale *et al.*, 1990a,b; Kim *et al.*, 2004; Kumamaru *et al.*, 1998; Misawa *et al.*, 2002; Pothuluri *et al.*, 1995, 1998a,b, 1999; Rogers and Hale, 1987; Suenaga *et al.*, 2001, 2002). A large volume of pathway information is available at the University of Minnesota biocatalysis/biodegradation database (http://umbbd.ahc.umn.edu/). However, many of the pathways and toxicity of the intermediates are unknown. The effect of environmental factors on the microbial ability to degrade organic pollutants remains uncertain. Our approach using SR-based IR spectromicroscopy to study biodegradation of organic pollutants by intrinsic microorganisms is less fundamental but more applied in nature. Questions to be addressed include whether the microorganisms are capable of decomposing the organic pollutants, and what the geochemical factors that affect the bioavailability of the organic pollutants to the microorganisms are. For microorganisms that degrade organic pollutants via a known pathway, we would also address if the intermediates are persistent and/or harmful to ecological receptors (Holman *et al.*, 2002b).

Finally, it is important to realize that information derived from SR-FTIR spectromicroscopy is only the tip of an iceberg of information. Because of the complexity of a biogeochemical system, this information alone is not sufficient for a thorough understanding of how intrinsic microorganisms transform pollutants and what factors could alter the microbial ability to transform the pollutants. It is also not sufficient for making reliable predictions of the potential risks of these pollutants and intermediates to ecological receptors and humans. The use of multiple complementary biochemical, analytical, and imaging techniques is necessary. A good example of the use of complementary techniques is the collaborative study by researchers from the Lawrence Berkeley National Laboratory (USA) and from the Georgian Academy of Science (Republic of Georgia) of chromium reduction by basalt-inhabiting aerobes (Abuladze *et al.*, 2002; Asatiani *et al.*, 2004; Holman *et al.*, 2004; Kalabegishvili *et al.*, 2003; Tsibakhashvili *et al.*,

2002a,b, 2004). In addition to the use of SR-FTIR spectromicroscopy to track the sequential reduction of chromium, they also used sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to identify chromium-induced changes in cell wall protein composition (Abuladze *et al.*, 2002), capillary electrophoresis to determine the effect of cell wall proteins on the mobility of chromium through cell wall (Tsibakhashvili *et al.*, 2002a), electron spin resonance (ESR) to determine/confirm chromium speciation in bulk cells (Kalabegishvili *et al.*, 2003), and micro-X-ray fluorescence analysis (μ–XRF) and micro-x-ray absorption fine structure (μ–XAFS) imaging of Cr, Fe, and Mn distribution. Scanning electron microscopy and transmission electron microscopy were also employed (Holman *et al.*, 2004). Such synergistic use of an array of different analytical and imaging techniques has allowed these researchers to discover the unexpected accumulation and immobilization of stable and toxic chromium intermediates by microorganisms, which will have significant implications in the applications of intrinsic microorganisms to remediate Cr(VI)-polluted earth and environmental materials.

III. BIOGEOCHEMICAL PROCESSES MEASURED BY SR-FTIR SPECTROMICROSCOPY

The measurement and imaging of biogeochemical processes by means of SR-FTIR spectromicroscopy involves the use of visible light and reflecting optics to view a magnified image of the sample and to select a microscopic surface area on the sample for IR reflection–absorption spectroscopic analysis. In this section, three biogeochemical studies conducted at the ALS are highlighted, following the description of instrumentation and spectral analysis. Interested readers are directed to read applications in other related biological, biogeochemical, and environmental areas (Benning *et al.*, 2002, 2003, 2004a,b; Bonetta *et al.*, 2002; Bradley *et al.*, 2005; Dokken *et al.*, 2005a,b; Facciotti *et al.*, 2001; Foriel *et al.*, 2004; Ghosh *et al.*, 2001; Vogel *et al.*, 2002, 2004; Yee and Benning, 2002; Yee *et al.*, 2003, 2004a,b; Yu, 2005a,b; Yu *et al.*, 2003, 2004).

A. INSTRUMENTATION

The instrumentation at beamline 1.4.3 at the ALS is similar to FTIR spectromicroscopy systems that are commercially available, except that the thermal source is replaced by an IR beam from the synchrotron (Fig. 7). Additionally, the beam is also passed through a beam position-locking
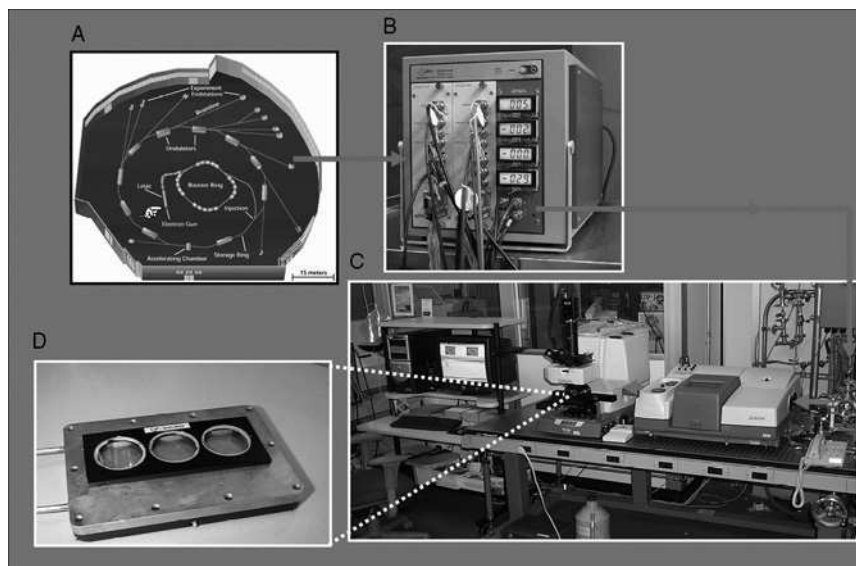
Figure 7 The beam position-locking system instrumentation at beamline 1.4.3 at the ALS. It is specifically made for probing biogeochemical processes *in situ* and *in vivo*. The beam from the synchrotron (A) is passed through the beam position-locking system (B) and then enters the commercially available FTIR spectromicroscopy system (C). During the experiment, samples are kept inside a stage mini-incubator (D). The addition of the beam-locking system is exceedingly helpful when studying biogeochemical materials that often have fine and highly heterogeneous surface features.

system (McKinney *et al.*, 2000; Scarvie *et al.*, 2004) to minimize the effect of the beam motion. Without this system, the beam tends to move on the sample during data acquisition for a variety of reasons. Such movements can be as large as several microns and cause artifacts and/or noise in the data. The addition of the beam-locking system is exceedingly helpful, when studying biogeochemical materials that often have fine and highly heterogeneous surface features.

The samples are maintained inside a mini-incubator, which is mounted on the microscope stage. The sample is positioned using a computer-controlled x-y-z stage with 0.1-μm precision allowing mapping measurements of FTIR spectra (through the incubator's ZnSe window) as a function of x- and y-position on the sample. The selection of the area is relatively subjective and relies on the geometry, color, crystallographic properties, and other material-specific features of the sample surface. Once the sample area is selected, the spectroscopic information of the selected surface area can be recorded *in situ* in a reflection mode.

B. Spectral Analysis

Because of the complexity of a biogeochemical system, one of our key efforts has been to carefully determine IR spectral features that are truly the molecular markers of the biogeochemical phenomena to be investigated. IR spectra of biomolecules in microbes (Choo-Smith *et al.*, 2001; Helm and Naumann, 1995; Helm *et al.*, 1991a,b; Kirschner *et al.*, 2001; Labischinski *et al.*, 1989; Maquelin *et al.*, 2002, 2003; Naumann *et al.*, 1982, 1988, 1996; Ngo-Thi *et al.*, 2003; Schultz and Naumann, 1991; Schultz *et al.*, 1987; Vandermei *et al.*, 1993, 1996) of many relevant minerals (Arnold and Wagner, 1988; Beran *et al.*, 1993; Collins, 1991; Delineau *et al.*, 1994; Eyring and Wadsworth, 1956; Farmer, 1974; Ha *et al.*, 1991; Keller *et al.*, 1952; Kretzschmar *et al.*, 1993; Luys *et al.*, 1982; Mielczarski *et al.*, 1993; Nguyen *et al.*, 1991; Plesko *et al.*, 1992; Povarennykh, 1978; Rossman and Aines, 1991; Salisbury *et al.*, 1991; Vilas *et al.*, 1994; White, 1971) and common environmental pollutants (Abdullah *et al.*, 2003; Bauschlicher, 1998a,b; Bauschlicher and Bakes, 2000; Bauschlicher and Langhoff, 1998; Bernstein *et al.*, 2005; Carrasco-Flores *et al.*, 2004, 2005; Chauhan *et al.*, 2004; Griffith *et al.*, 1959; Hawkins *et al.*, 1955; Hudgins and Sandford, 1998a,b,c; Hudgins *et al.*, 2000; Humphrey, 1961; Janni *et al.*, 1997; Jensen, 2004a,b; Jensen and Jensen, 2004; Li *et al.*, 2004; Ludwig *et al.*, 2000; Mattioda *et al.*, 2002; Pauzat and Ellinger, 2001, 2002; Ruiterkamp *et al.*, 2002; Seelenbinder and Brown, 2002; Todd *et al.*, 2002; Zhang *et al.*, 2005) are already well-known with specific peaks and groups of peaks that can be related to specific biochemical and chemical groups of single molecules in an ideal system. The traditional approach of spectral analysis, which is intended to identify particular compounds, involves a band-shape analysis followed by direct assignment of characteristic absorption bands in the IR spectrum. However, in a complicated and often transient biogeochemical system under *in situ* and *in vivo* conditions, these specific peaks and bands of peaks may shift, and the overall pattern may even change and deviate from the well-established features. To date, our general approach has focused on a small number of important spectral features that could be derived from a series of simplified model systems prior to the SR-FTIR spectromicroscopy experiment. For time-course experiments, we would also combine the traditional direct assignments and the difference spectroscopy approach to guide the interpretation of the absorption bands as a function of exposure time. We evaluated the intensity of each absorption band by means of the method of the most probable baseline (Lijour *et al.*, 1994). It is important to note that as the beam current of the synchrotron decreases with time between electron refills, the beam intensity decreases proportionally, which needs to be taken into account if one wants to accurately measure absorption band intensity.

We have found that rescaling the intensity of the absorption bands by means of an internal-standard equivalent approach works reliably.

C.   APPLICATION EXAMPLES

With the completion of sequencing of genomes of many organisms and the continuous success in identifying gene products (proteins) and metabolic pathways, one of the central interests in biogeochemical and environmental research is to apply this wealth of information to understand and to design appropriate strategies to utilize metabolic capabilities in living microorganisms to remediate pollutants in earth and environmental materials. The success of these directions will ultimately be determined by how well one can measure without disturbing the relevant dynamic processes in a biogeochemical system, for example, the redox transformations of heavy metals by metal-reducing bacteria, or degradation of carcinogenic organic pollutants. These examples will illustrate how SR-FTIR spectromicroscopy can be a useful tool that allows one to get a step closer to achieve this important goal.

1.   Reduction of Hexavalent Chromium by Basalt-Inhabiting Aerobes

Compounds containing chromium atoms can be potentially hazardous contaminants in the environment. The degree of the hazard depends on the chemical state of the chromium in the compounds in which it occurs. Chromium at its hexavalent state [Cr(VI)] is usually highly soluble in water and therefore mobile in the environment, so the contamination spreads, and it is toxic and suspected to be carcinogenic. However, chromium at its trivalent state [Cr(III)] is relatively insoluble in water and significantly less harmful. Geochemical and biogeochemical processes that convert chromium from the hexavalent to the trivalent state are potentially useful for environmental remediation. We demonstrated the use of SR-FTIR spectromicroscopy to illustrate that certain bacteria found naturally in basalt are effective agents in the "biogeochemical" transformation of chromium from the undesirable hexavalent state to the less harmful trivalent state, thereby resolving an on-going controversy about the nature of the conversion. (Holman *et al.*, 1999).

This is the first time that biogeochemical transformation of Cr(VI) by microorganisms on a mineral surface has been nondestructively monitored and studied where it occurs. Distinct and relevant IR absorption bands

(Table I) were used as chemical markers to detect the presence of microorganisms and identify different chromium species on specimen surfaces. In addition, the brightness of the IR radiation from the synchrotron IR beamline makes spatially resolved spectroscopy (spectromicroscopy) possible for imaging biogeochemical systems.

Two reduction mechanisms in polluted geological materials have previously been postulated for the reduction of Cr(VI) compounds. The biological mechanism requires the presence of microorganisms to aerobically reduce the Cr(VI). The chemical mechanism relies on metal oxides, such as Fe(II) compounds, to catalyze the Cr(VI)-reduction reaction. We conducted synchrotron FTIR spectromicroscopy experiments to distinguish the relative significance of these two mechanisms. In addition, we evaluated the effects of common organic cocontaminants, such as toluene vapor, on the biotic reduction process (Fig. 10).

For magnetite surfaces of mixed iron oxides that contain no living microorganisms, a 5-day exposure to Cr(VI) compounds resulted in statistically insignificant changes in the IR chemical markers, indicating that little catalysis of Cr(VI) reduction was occurring. On samples with living microorganisms, however, some Cr(VI) reduction was detected (Fig. 8). Moreover, when the samples with living microorganisms were incubated in dilute toluene vapor, statistically significant changes in both IR-absorption intensity and characteristic band shapes were observed for Cr(VI), as were new bands signaling the existence of intermediate Cr(V). FTIR spectromicroscopy showed that the changes in the IR absorption bands occurred at the sites of bacterial concentration. Measured images of the surface at characteristic absorption bands showed a strong correlation between peak depletion of Cr(VI) and depletion of toluene and peak concentration of biological molecules (Fig. 9).

Table I
Spectral Regions and Distinct Absorption Bands Within Each Region for Microorganisms (Including Bacteria), Cr(VI)-, Cr(V)-, and Cr(III)-Compounds, Toluene, and Catechols in Mineral/Microorganisms/Cr/Toluene System (Holman *et al.*, 1999) (Reproduced with permission from *Geomicrobiol. J.*, 1999, 16, 307–324. Copyright 1999 Taylor & Francis.)

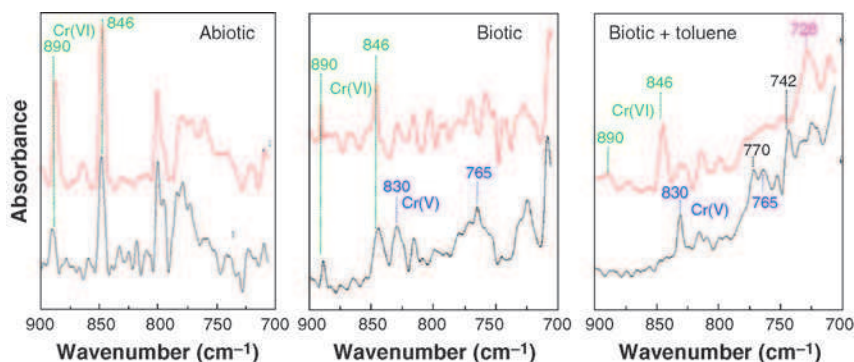| Compounds	Spectral regions (cm^{-1})	Absorption bands (cm^{-1})
Microorganisms (protein)	1800–1500	~1650; ~1550
Cr(VI) compounds	900–800	~846; ~890
Cr(V) compounds	900–700	~830; ~764
Cr(III) compounds	850–750	~810; ~798
Toluene	800–650	~728; ~695
Catechols	800–700	~770; ~742

Figure 8 SR-FTIR spectra of chromate on magnetite surfaces during the 5-day experiment of (left) abiotic reduction, (middle) biotic reduction in the absence of other organic compounds, and (right) biotic reduction in the presence of toluene vapor (as a model volatile organic compound). (—) $t < 1$ day, shifted vertically for visual clarity. (—) $t = 5$ days. Although the total chromate concentration for each of the three experiments was the same, microbial-mineral surface roughness and redistribution during evaporation results in heterogeneous spatial distributions of Cr(VI) concentrations. The most relevant vibrational frequencies identified are marked: 890 and 846 cm^{-1} correspond to Cr(VI), 830 and 765 cm^{-1} correspond to Cr(V), 770 and 742 cm^{-1} are catechols, and 728 cm^{-1} is toluene. We observe that microbial reduction of Cr(VI) is the dominant mechanism in our experimental system. The microbial chromium reduction is further enhanced during the microbial degradation of the organic compound toluene (Holman *et al.*, 1999). (Reproduced with permission from *Geomicrobiol. J.*, 1999, **16**, 307–324. Copyright 1999 Taylor & Francis.) (See Color Insert.)

In a study to determine if this microbial reduction process could occur in real geological samples, composite mineral surfaces of basalt rock chips containing resident communities of microbes were exposed to solutions of Cr(VI) and toluene vapor. At the end of 4 months, FTIR spectromicroscopy showed that Cr(VI)-tolerant and Cr(VI)-reducing natural microorganisms were thriving in association with Cr(III) (Fig. 10). The reduced Cr(III) state was confirmed by XAFS spectroscopy at ALS beamline 10.3.2 (Fig. 11). The nondestructive IR spectromicroscopy studies, combined with XAFS spectroscopy and microbiological techniques, show that highly mobile and toxic Cr(VI) contaminants can be biologically reduced into less soluble, less toxic Cr(III) compounds. The FTIR method can now be expanded to examine other IR-amenable microbial/chemical contaminant systems.

2. Mycobacterial Metabolization of Pyrene in Humic Acid

Contaminants in the environment come in many forms, one of which is the relatively recalcitrant toxic organic (carbon-based) family of
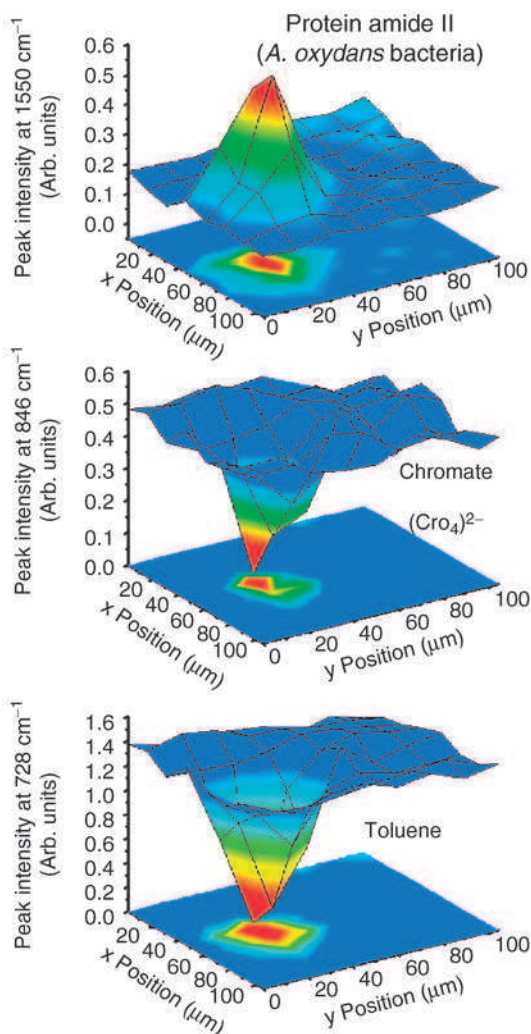
Figure 9 During the 5-day study period, *Arthrobacter oxydans* bacteria (isolated from the basalt core sample) attached themselves to magnetite surfaces. They reduced Cr(VI) while degrading toluene. SR-FTIR spectromicroscopy measurements at the end of the experiment show the spatial distribution of (top) *A. oxydans*, (middle) chromate, and (bottom) toluene, as measured by their spectral signatures (Holman *et al.*, 1999). (Reproduced with permission from *Geomicrobiol. J.*, 1999, **16**, 307–324. Copyright 1999 Taylor & Francis.)

chemicals known as polycyclic aromatic hydrocarbons (PAHs). These include more than 100 different chemicals resulting from incomplete burning of coal, oil and gas, garbage, or other organic substances like
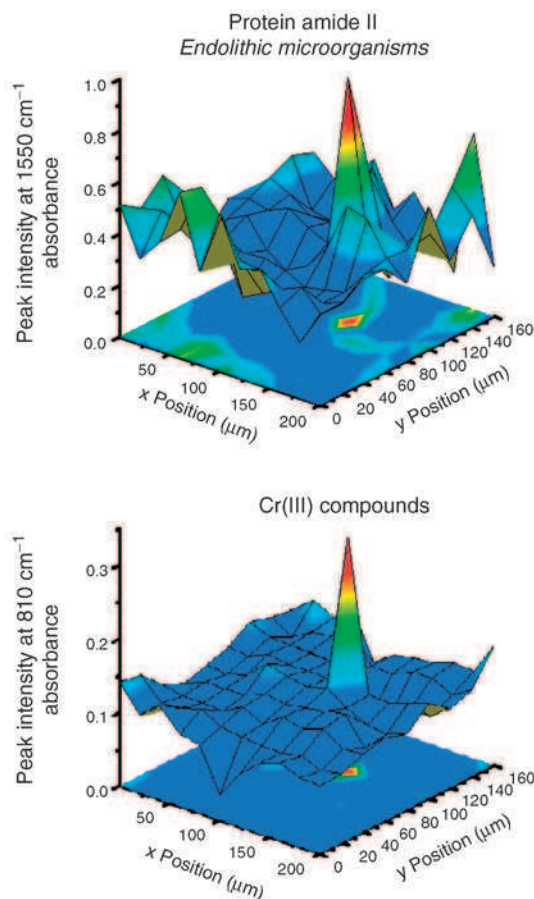
Figure 10 Distribution of indigenous endolithic microorganisms (top) and the Cr(III) compounds (bottom) as measured by SR-FTIR spectromicroscopy at the end of the 4-month Cr(VI)–microbe–basalt experiment. Only chromium-tolerant and chromium-reducing microorganisms proliferated during the study period (Holman *et al.*, 1999). (Reproduced with permission from *Geomicrobiol. J.*, 1999, **16**, 307–324. Copyright 1999 Taylor & Francis.)

tobacco or grilled meat. Converting PAHs into nontoxic chemicals removes the hazard, but learning how to do this in an efficient and cost-effective way remains to be accomplished. Here we made use of synchrotron infrared spectromicroscopy to show that the speed of biodegradation can be dramatically increased (by almost a 100-fold) by adding a certain soil-derived organic (humic) acid along with the bacteria to a PAH spot on a mineral surface. (Holman *et al.*, 2002b).
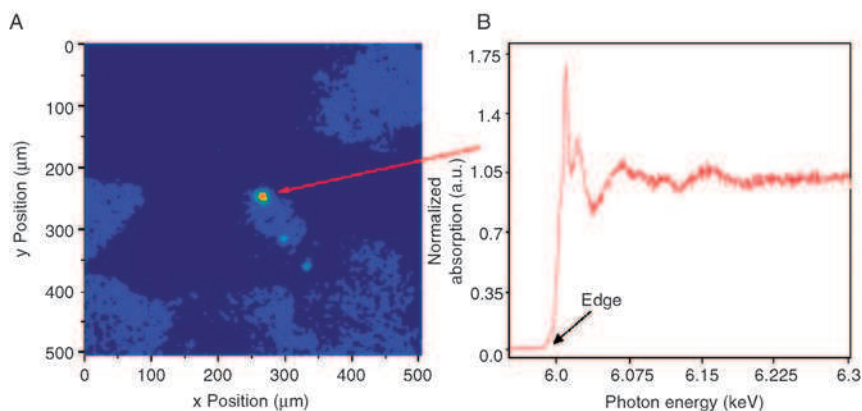
Figure 11 Confirmation of Cr (III) oxidation state by micro-X-ray analysis on the similar area of the identical sample studied by SR-FTIR (see Fig. 5). (A) Chromium elemental mapping by micro-X-ray fluorescence analysis (μ-XRF). The colors go from black (chromium concentration below detection limit) to red (high chromium concentration). (B) Average of nine micro-X-ray absorption fine structure (μ-XAFS) scans taken at the highest concentration spot shows no Cr(VI) preedge peak and is consistent with Cr(III) compounds. Each data point represents 20 s counting time. The energy increments are 0.5 eV (Holman *et al.*, 1999). (Reproduced with permission from *Geomicrobiol. J.*, 1999, **16**, 307–324. Copyright 1999 Taylor & Francis.)

The role of humic acid (HA) in the biodegradation of toxic PAHs has been the subject of controversy, particularly in unsaturated environments. By utilizing an IR Fourier transform spectromicroscope and a very bright, nondestructive synchrotron photon source (SR-FTIR spectromicroscopy), we monitored *in situ* and over time the influence of HA on the degradation of pyrene (a model PAH) by a bacterial colony on a magnetite surface. Our results indicate that HA dramatically shortens the onset time for PAH bio-degradation from 168 to 2 h. These results will have significant implications for the bioremediation of contaminated soils.

The pyrene-degrading bacterium used for this study is *Mycobacterium* sp. JLS (Fig. 12), a gram-positive, rod-shaped bacterium isolated from PAH-contaminated soil at the Libby groundwater superfund site in Libby, Montana, USA. Abiotic (no bacteria present) results (inserts in Fig. 13A and B) show that almost all of the pyrene remains on the mineral surface for the duration of the study, owing to slow removal mechanisms. After intro-duction of *M*. sp. JLS in the absence of HA, it took the bacteria about 168 h to produce sufficient glycolipids to solubilize pyrene. At this point, bio-degradation could proceed, resulting in a rapid decrease of pyrene and a rapid increase of biomass within the next 35 h. After the pyrene was depleted, the biomass signal significantly decreased, presumably as the *M*. sp. JLS
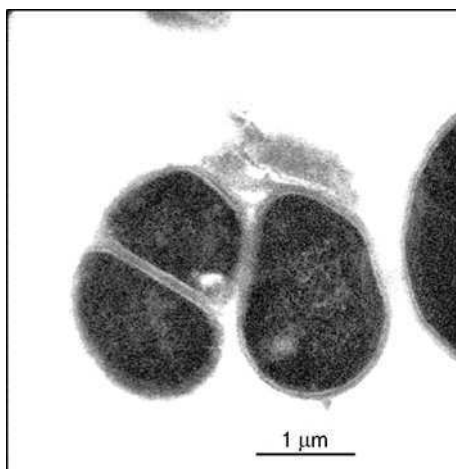
Figure 12 A transmission electron microscopy (TEM) image of the newly isolated gram-positive cocci *Mycobacterium* sp. JLS (GenBank accession no. AF387804). It appears that *M.* sp. JLS degrades polycyclic aromatic hydrocarbons, such as pyrene via a novel pathway. However, it gained biomass rapidly while degrading the compounds (Holman *et al.*, 2002b). Time-resolved analysis of spectra from SR-FTIR spectromicroscopy did not reveal fingerprints of known metabolites. This is further confirmed by follow-up mass spectrometry analysis of the sample. (Figure courtesy of W. R. Sims.)

bacteria transformed themselves into ultramicrocells, a starvation-survival strategy commonly observed among bacteria in oligotrophic environments.

In the presence of HA, pyrene biodegradation began within an hour, and the pyrene was depleted by the end of the fourth hour, with a concurrent increase of biomass (Fig. 13B). Both the degradation of pyrene and the increase of biomass corroborate the effectiveness of Elliott soil humic acid (ESHA) in radically accelerating biodegradation of pyrene. It is likely that the water-insoluble pyrene is solubilized into the cores of ESHA pseudomicelles and, therefore, becomes directly available for bacterial uptake and consumption.

Over longer times, the remaining IR absorption bands of pyrene on magnetite surfaces first showed a slight increase and subsequently a decrease. The increase is probably due to diffusion of pyrene trapped in micropores (<0.5 μm in diameter) of the magnetite and/or neighboring surfaces of higher pyrene concentration after the first wave of rapid depletion of pyrene by *M.* sp. JLS set up a diffusion gradient from the pyrene-containing micropores toward the bacterial colony. For the surface containing HA, the biomass remained almost constant over a period of more than 200 h, indicating that the flux of pyrene from the micropores was sufficient to
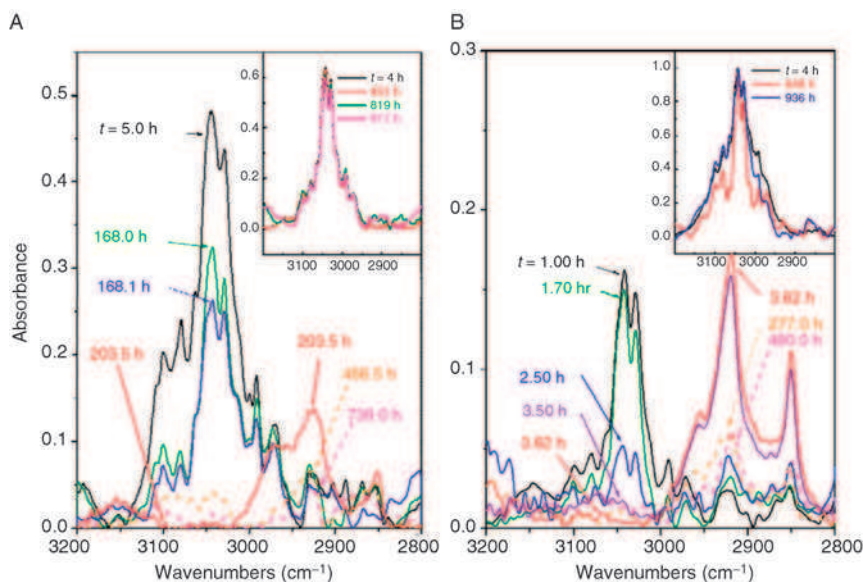
Figure 13 Time series of SR-FTIR absorption bands corresponding to pyrene and biomass formation following the degradation of pyrene by *M.* sp. JLS on magnetite surfaces. Panels A and B are from a sample free of and with ESHA. The time at which each spectrum as acquired is labeled. They show the transient behavior of pyrene doublet at 3044 and 3027 cm^{-1} and biomass IR absorption bands at 2921 and 2850 cm^{-1}. Similar behavior was observed for pyrene absorption band centered at 1185 cm^{-1}. Inserts are time series from abiotic control experiments (Holman *et al.*, 2002b). (Reproduced with permission from *Environ. Sci. Technol.*, 2002, **36**, 1276–1280. Copyright 2002 Am. Chem. Soci.)

maintain the bacterial colony. For the surface free of HA, there is little evidence of the presence of a quasisteady state biomass (Fig. 14).

At the end of the time-resolved experiment (about 460 h), spatial distributions of pyrene, *M.* sp. JLS, and ESHA were measured by acquiring IR spectra at 5-µm intervals across the center of the bacterial colony with HA. Figure 15 shows contour maps of the spatial distribution of measured IR absorbance corresponding to *M.* sp. JLS, HA, and pyrene. The central region of the maps has a high-population density of *M.* sp. JLS and a high concentration of HA, but the pyrene in this region was completely biodegraded. Where pyrene is present without *M.* sp. JLS, there is no significant degradation.

We conclude that SR-FTIR spectromicroscopy can assess real-time interactions between multiple constituents in contaminated soils. Combined with conventional mineralization measurements, which monitor respiration through carbon dioxide production, SR-FTIR spectromicroscopy is
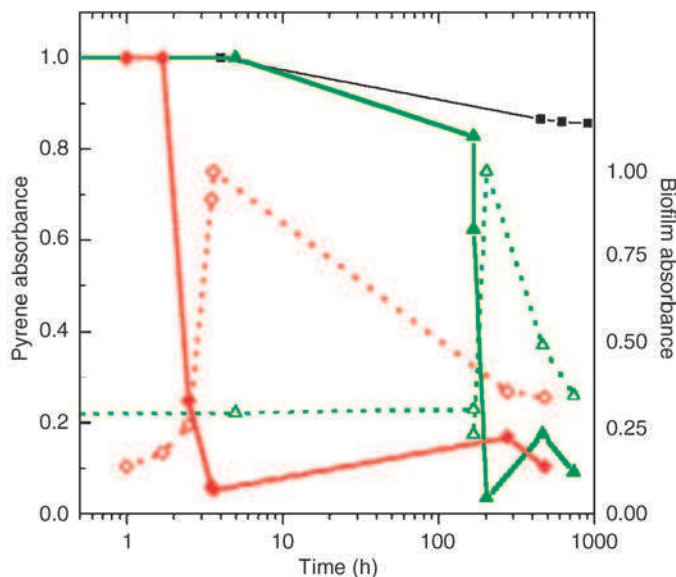
Figure 14 Summary of the SR-FTIR results showing that pyrene degradation occurs much faster when ESHA is present (note the log scale on the time axis). The pyrene absorbance was measured at 1185 cm^{-1} and biomass IR absorption band at 2921 cm^{-1}. The color scheme is black for abiotic, green for biotic without ESHA, and red for biotic with ESHA. The solid lines show the pyrene amount as a function of time for each experiment. The dotted lines show a subsequent increase in *M.* sp. JLS biomass after pyrene degradation (Holman *et al.*, 2002b). (Reproduced with permission from *Environ. Sci. Technol.*, 2002, **36**, 1276–1280. Copyright 2002 Am. Chem. Soci.)

thus a powerful tool for evaluating bioremediation options and designing bioremediation strategies for contaminated vadose zone environments.

3. Rapid Screening for Remediation Capability of a Microbial Community

Can infrared light from a synchrotron be used to screen for metabolic activities in a living microbial community that can degrade organic pollutants? If so, it would open up possibilities for the eventual use of synchrotron infrared light in environmental diagnostics or environmental health research. The experiment summarized here is an infrared imaging of transformation of toluene by a microbial community on vesicular basalt surfaces. Our preliminary results suggest that some day
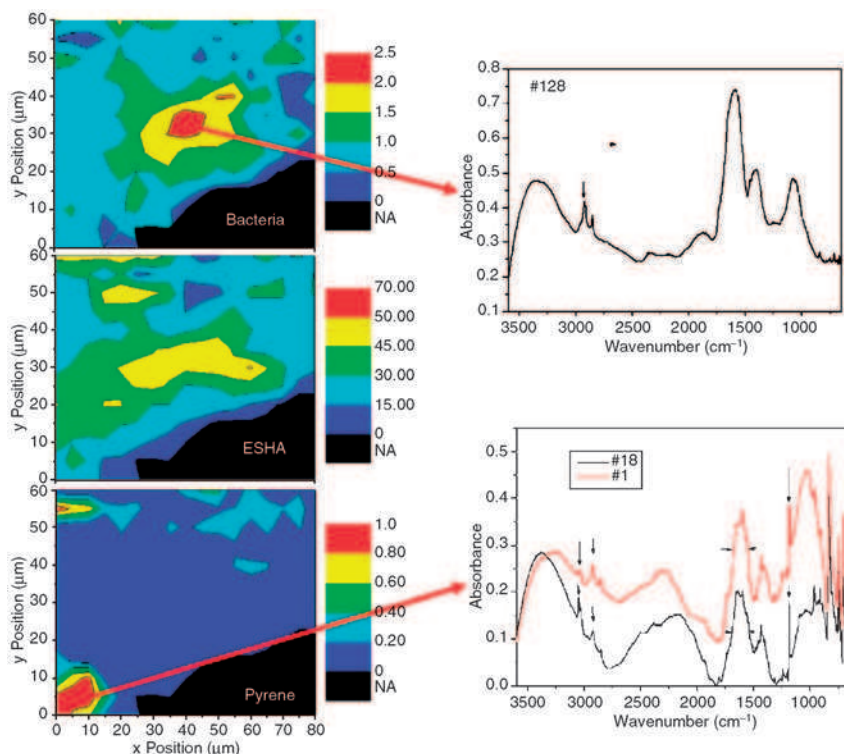
Figure 15 Contour diagrams from IR mapping obtained at the end of the experiment, showing the spatial distribution of the IR absorption peaks corresponding to (top) *M*. sp. JLS bacteria, (middle) ESHA, and (bottom) pyrene. Appropriate spectral regions were integrated for each point on the maps. The color scales for each contour plot are red for high integrated IR peak area (high concentration of the corresponding component) and blue for low peak area (low concentration); black is an out-of-focus region of the sample. The center of the map shows a region with high density of bacteria and high concentration of ESHA, where pyrene has been completely degraded (Holman *et al.*, 2002b). Note that the quality of the spectra is excellent even on such complicated surfaces of earth materials (Arrows are pointing at some of marker peaks employed in this study.) (Holman *et al.*, 2002b). (Reproduced with permission from *Environ. Sci. Technol.*, 2002, **36**, 1276–1280. Copyright 2002 Am. Chem. Soc.)

it may be routine to study a tiny microbial colony, by using synchrotron infrared spectroscopy, and to screen for microbes and conditions that are most effective in detoxifying environmental pollutants. (Holman and Geller, 2005).

The possibility of utilizing the capability of intrinsic microorganisms to decompose and even mineralize organic pollutants has stimulated intensive interests in exploring if these biotransformation reactions actually take place on surfaces of geologic materials. Conceptual and technological

improvements in environmental microbiology have advanced our ability to
partly address these issues. For example, the use of the DNA probes for
specific enzymatic activities enables researchers to determine if certain genes
are present in the bulk microorganisms that can initiate and sustain the
desirable transformation of pollutants (Koenigsberg *et al.*, 2005). Detection
of unique intermediate metabolites in site-derived samples provides evidence
for the occurrence of *in situ* contaminant biotransformation. Together with
microcosm experiments they globally address the questions of whether or
not the bacteria interact with contaminants. However, these efforts are labor
intensive and time-consuming. We are conducting a feasibility study to
evaluate if SR-FTIR spectromicroscopy can be an ideal screening tool to
rapidly identify microbial remedial capability on mineral surfaces.

   The geological sample used was a fragment of a vesicular basalt rock from
a site formerly polluted with volatile organic compounds (VOCs). The
sample was exposed to 100 ppm of toluene vapor at 100% relative humidity
for 5 days. Distinct and relevant IR absorption bands of toluene and its
metabolites from a common degradation pathway (Fig. 16) are used to mark
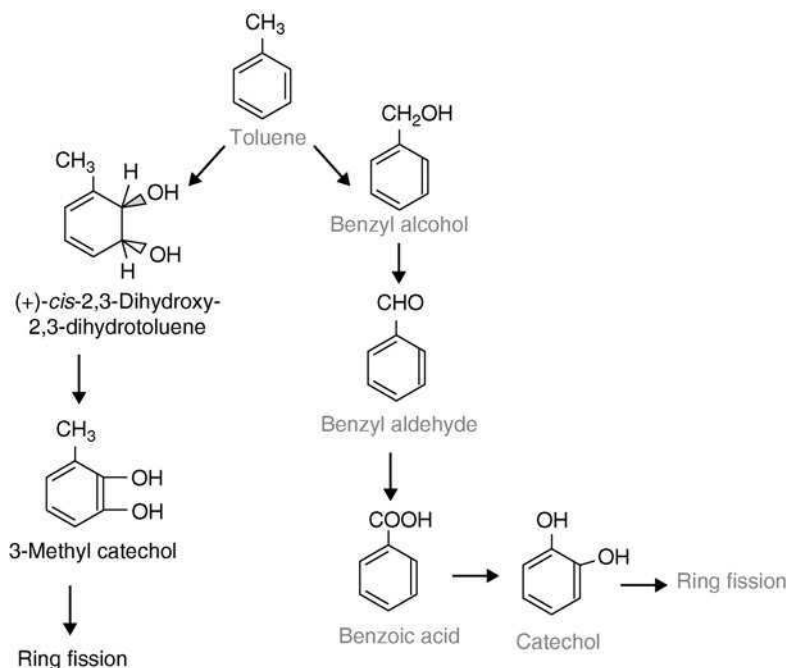
Figure 16   The possible pathway for the metabolic degradation of toluene by the intrinsic
microbial communities in the earth materials. Due to matrix interference, we only tracked the
marker peaks for toluene, benzyl alcohol, benzoic acid, and catechol in this study (see Table II).

the progression and capability of toluene degradation (Table II). At the end of the fifth day, chemical images from SR-FTIR spectromicroscopy showed that the native microorganisms were thriving in association with various capabilities of toluene degradation (Fig. 17). This demonstrates that the excellent spatial resolution of SR-FTIR spectromicroscopy provides a means for determining the degree to which the toxic toluene was metabolized by the microorganisms.
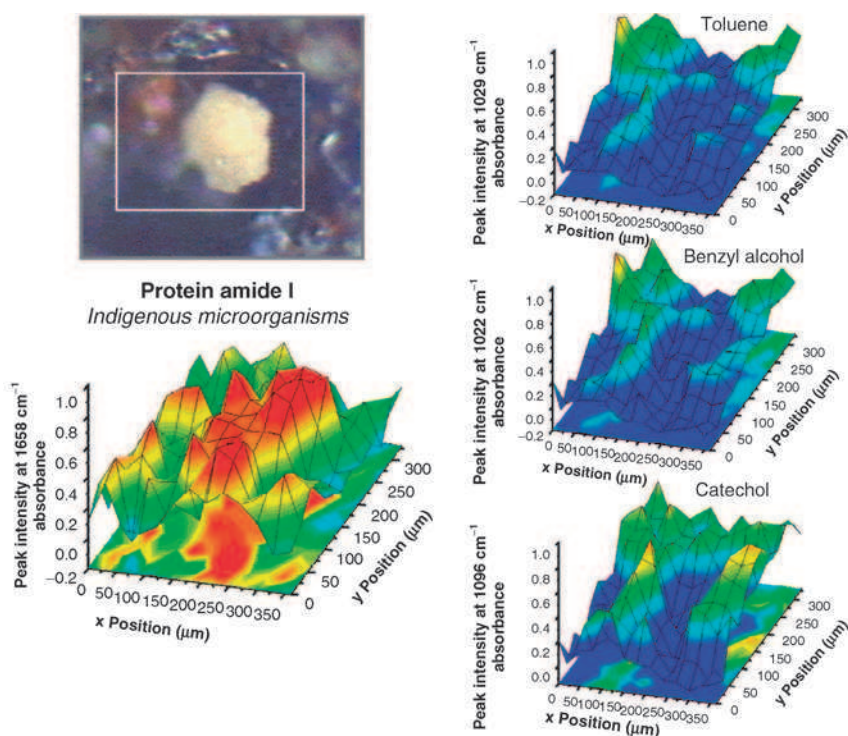

Figure 17 Chemical images from SR-FTIR spectromicroscopy showed that the native microorganisms were thriving during toluene degradation. Spectral images were rotated clockwise and tilted (relative to the bright-field micrograph) for clarity. A bright-field micrograph of microbial colonies formed on the basalt surfaces after exposure to 100-ppm toluene vapor for 5 days (left top). The spatial distribution of IR absorption peaks corresponding to (left bottom) indigenous microorganisms, (right top) toluene, and the metabolites (right middle and bottom). It appears that many native microbes metabolized nearly all the toluene immediately with some accumulation of the nontoxic metabolites benzyl alcohol and catechol. No accumulation of benzoic acid was detected. This implies that intrinsic microbial communities at the former polluted site remained efficient in detoxifying toluene (unpublished data).

Table II
Spectral Regions and Distinct Absorption Bands Within Each Region for Microorganisms (Including Bacteria), Toluene, Benzoic Acid, and Catechols in Basalt/Microorganism/Toluene System (Holman and Geller, 2005)

Compounds	Spectral regions (cm^{-1})	Absorption bands (cm^{-1})
Microorganisms (protein)	1800–1500	~1658; ~1548
Toluene	1250–650	~1029; ~728; ~695
Benzyl alcohol	1250–650	~1200; ~1022
Benzoic acid	1250–650	~930
Catechols	1250–700	~1096; ~770; ~742

IV.   FUTURE POSSIBILITIES AND REQUIREMENTS

Although SR-FTIR spectromicroscopy is an emerging analytical and imaging technology for studying biogeochemical processes *in vivo* and *in situ*, considerable experience has already been obtained in its use in evaluating microbial interactions with environmental pollutants. It seems as if only a small part of this noninvasive technology has been explored to date. For example, the quantitative capability of IR spectroscopy for accurately quantifying the transformation of metal ions or organic substrates, for defining the interrelationship between such transformation and metabolic activities, and even for measuring the chemical or activity gradient and thus the chemical fluxes across a microbial colony have not been fully utilized. Such utilities can be enhanced by a number of emerging or hoped-for advances in other relevant technologies. Improved software for the automated and accurate analysis of the spectra will make accurate quantitation easier.

Improved experimental systems are also essential. To date, the major experimental obstacles lie not in the synchrotron IR instruments themselves. Instead, they lie in two difficulties: (1) in rapidly controlling the optimum conditions for experiments before products of microbial functions are measured, and (2) in optimizing immediate data processing and interpretation. The existing techniques are relatively time consuming and labor intensive. Their fragility frequently results in major losses of sample and experimental time, and they require many steps that can take days to achieve the optimum experimental conditions.

Additionally, the future utility of the technique will also be enhanced by combining SR-FTIR spectromicroscopy with other techniques of higher specificity. For example, the most popular approach that is beneficial to

this combination appears to be the visible/IR imaging along with fluorescence microscopy. While visible imaging provides information on the physical features of the biogeochemical system and IR imaging yields global chemical information of the system, fluorescence microscopy allows one to observe localized environments (e.g., redox conditions, molecular cluster dimensions) (Kilkenny *et al.*, 2002; Rocheleau *et al.*, 2002; Zhang *et al.*, 2002) and key-dynamical processes that govern the function and structure of cells (Kahng and Shapiro, 2003; Thanbichler *et al.*, 2005; Viollier and Shapiro, 2004; Viollier *et al.*, 2002; Weijer, 2003). The fluorescent probes can be endogenous molecules such as NAD(P)H (Latouche *et al.*, 2000; Piston and Knobel, 1999; Simon *et al.*, 1996), genetically encoded specific fluorescent proteins (Orser *et al.*, 1995; Vandyk *et al.*, 1995), or passive markers of specific fluorescent molecules/dyes (Haugland, 2002). By establishing an associative analysis that links the genetically encoded molecules and marked cellular events from fluorescence microscopy to the global chemical information derived from SR-FTIR spectromicrosocpy, one can truly expand the existing understanding of biogeochemical capabilities in living microbes and developing biotechnologies for utilizing such capabilities for the benefit of environmental management.

ACKNOWLEDGMENTS

REFERENCES

Abdullah, H. H., Kubba, R. M., and Shanshal, M. (2003). Vibration frequencies shifts of naphthalene and anthracene as caused by different molecular charges. *Z. Naturforsch. A* **58**, 645–655.

Abuladze, M. K., Asatiani, N. V., Bakradze, N. G., Kartvelishvili, T. M., Holman, H.-Y. N., Kalabegishvili, T. L., Mosulishvili, L. M., Rcheulishvili, A. N., Sapojnikova, N. A., and Tsibakhashvili, N. Y. (2002). Effect of chromium action on the protein composition of Arthrobacter oxydans. *Fresen. Environ. Bull.* **11**, 562–567.

Aksu, Z. (2005). Application of biosorption for the removal of organic pollutants: A review. *Process Biochem.* **40**, 997–1026.

Amend, J. (2004). Geomicrobiology and genomes. *Geotimes* **49**, 40–41.

Amonette, J. E., Heald, S. M., and Russell, C. K. (2003). Imaging the heterogeneity of mineral surface reactivity using Ag(I) and synchrotron X-ray microscopy. *Phys. Chem. Miner.* **30**, 559–569.

Andrejchuk, V. N., and Klimchouk, A. B. (2001). Geomicrobiology and redox geochemistry of the karstified Miocene gypsum aquifer, western Ukraine: The study from Zoloushka Cave. *Geomicrobiol. J.* **18**, 275–295.

Arnesano, F., Banci, L., Bertini, I., Mangani, S., and Thompsett, A. R. (2003). A redox switch in CopC: An intriguing copper trafficking protein that binds copper(I) and copper(II) at different sites. *Proc. Natl. Acad. Sci. USA* **100**, 3814–3819.

Arnold, G., and Wagner, C. (1988). Grain-size influence on the mid-infrared spectra of the minerals. *Earth Moon Planets* **41**, 163–171.

Asatiani, N. V., Abuladze, M. K., Kartvelishvili, T. M., Bakradze, N. G., Sapojnikova, N. A., Tsibakhashvili, N. Y., Tabatadze, L. V., Lejava, L. V., Asanishvili, L. L., and Holman, H.-Y. (2004). Effect of chromium(VI) action on *Arthrobacter oxydans*. *Curr. Microbiol.* **49**, 321–326.

Baker, B. J., and Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiol. Ecol.* **44**, 139–152.

Balkwill, D. L., and Ghiorse, W. C. (1985). Characterization of subsurface bacteria associated with 2 shallow aquifers in Oklahoma. *Appl. Environ. Microb.* **50**, 580–588.

Bantignies, J. L., Moulin, C. C. D., Dexpert, H., Flank, A. M., and Williams, G. (1995). Asphaltene adsorption on kaolinite—characterization by infrared microspectroscopy and X-Ray-absorption spectroscopy. *CR Acad. Sci. II* **320**, 699–706.

Barer, G. R. (1954). Quantitative measurements on spreading phenomena in skin. *Brit. J. Pharm. Chemoth.* **9**, 346–351.

Barer, R. (1949). The reflecting microscope in anatomical research. *J. Anat.* **83**, 73–74.

Barer, R. (1953). Determination of dry mass, thickness, solid and water concentration in living cells. *Nature* **172**, 1097–1098.

Barer, R., and Joseph, S. (1954). Refractometry of living cells.1. Basic principles. *Q. J. Microsc. Sci.* **95**, 399–423.

Barer, R., Cole, A. R. H., and Thompson, H. W. (1949). Infra-red spectroscopy with the reflecting microscope in physics, chemistry and biology. *Nature* **163**, 198–201.

Barker, W. W., and Banfield, J. F. (1996). Biologically versus inorganically mediated weathering reactions: Relationships between minerals and extracellular microbial polymers in lithobiontic communities. *Chem. Geol.* **132**, 55–69.

Barker, W. W., and Banfield, J. F. (1998). Zones of chemical and physical interaction at interfaces between microbial communities and minerals: A model. *Geomicrobiol. J.* **15**, 223–244.

Bauschlicher, C. W. (1998a). Infrared spectra of polycyclic aromatic hydrocarbons: Nitrogen substitution. *Chem. Phys.* **234**, 87–94.

Bauschlicher, C. W. (1998b). Infrared spectra of polycyclic aromatic hydrocarbons: Oxygen substitution. *Chem. Phys.* **233**, 29–34.

Bauschlicher, C. W., and Bakes, E. L. O. (2000). Infrared spectra of polycyclic aromatic hydrocarbons (PAHs). *Chem. Phys.* **262**, 285–291.

Bauschlicher, C. W., and Langhoff, S. R. (1998). Infrared spectra of polycyclic aromatic hydrocarbons: Methyl substitution and loss of H. *Chem. Phys.* **234**, 79–86.

Benison, G. C., Di Lello, P., Shokes, J. E., Cosper, N. J., Scott, R. A., Legault, P., and Omichinski, J. G. (2004). A stable mercury-containing complex of the organomercurial lyase MerB: Catalysis, product release, and direct transfer to MerA. *Biochemistry-US* **43**, 8333–8345.

Bennett, P. C., Hiebert, F. K., and Rogers, J. R. (2000). Microbial control of mineral-groundwater equilibria: Macroscale to microscale. *Hydrogeol. J.* **8**, 47–62.

Benning, L. G., Yee, N., Phoenix, V., and Konhauser, K. O. (2002). The *in situ* molecular characterisation of a biomineralization process: A synchrotron infrared study. *Geochim. Cosmochim. Acta* **66**(Suppl. 1), A68.

Benning, L. G., Phoenix, V., Yee, N., and Hammer, O. (2003). Bacterial-silica interactions: Affinity or fate? *Abst. Pap. Am. Chem. S.* **226**, U590.

Benning, L. G., Phoenix, V. R., Yee, N., and Konhauser, K. O. (2004a). The dynamics of cyanobacterial silicification: An infrared micro-spectroscopic investigation. *Geochim. Cosmochim. Acta* **68**, 743–757.

Benning, L. G., Phoenix, V. R., Yee, N., and Tobin, M. J. (2004b). Molecular characterization of cyanobacterial silicification using synchrotron infrared micro-spectroscopy. *Geochim. Cosmochim. Acta* **68**, 729–741.

Benzerara, K., Chapon, V., Heulin, T., Guyot, F., Menguy, N., Skouri, F., and Barakat, M. (2002). Geomicrobiology of carbonate precipitations at the surface of pyroxenes in an aridic soil. *Geochim. Cosmochim. Acta* **66**, A69.

Benzerara, K., Yoon, T. H., Menguy, N., Tyliszczak, T., and Brown, G. E. (2005). Nanoscale environments associated with bioweathering of a Mg-Fe-pyroxene. *Proc. Natl. Acad. Sci. USA* **102**, 979–982.

Beran, A., Langer, K., and Andrut, M. (1993). Single-crystal infrared-spectra in the range of Oh fundamentals of paragenetic garnet, omphacite and kyanite in an eklogitic mantle xenolith. *Miner. Petrol.* **48**, 257–268.

Bernstein, M. P., Mattioda, A. L., Sandford, S. A., and Hudgins, D. M. (2005). Laboratory infrared spectra of polycyclic aromatic nitrogen heterocycles: Quinoline and phenanthridine in solid argon and H2O. *Astrophys. J.* **626**, 909–918.

Bird, G. R., and Blout, E. R. (1952). Infrared microspectroscopy of biologic materials. *Lab. Invest.* **1**, 266–272.

Blout, E. R. (1953). Infrared microspectroscopy—instrumentation and some biological applications. *Trans. N. Y. Acad. Sci.* **15**, 280–281.

Blout, E. R., Bird, G. R., and Grey, D. S. (1949). Infrared microspectroscopy. *Anal. Chem.* **21**, 1585.

Bonetta, D. T., Facette, M., Raab, T. K., and Somerville, C. R. (2002). Genetic dissection of plant cell-wall biosynthesis. *Biochem. Soc. Trans.* **30**, 298–301.

Bradley, J., Dai, Z. R., Erni, R., Browning, N., Graham, G., Weber, P., Smith, J., Hutcheon, I., Ishii, H., Bajt, S., Floss, C., Stadermann, F., *et al.* (2005). An astronomical 2175 angstrom feature in interplanetary dust particles. *Science* **307**, 244–247.

Brenner, D. (1983). Infrared microspectroscopy of coals. *P. Soc. Photo-opt. Inst.* **411**, 8–12.

Brown, G. E., and Parks, G. A. (2001). Sorption of trace elements on mineral surfaces: Modern perspectives from spectroscopic studies, and comments on sorption in the marine environment. *Int. Geol. Rev.* **43**, 963–1073.

Burton, S. K., and Lappin-Scott, H. M. (2005). Geomicrobiology, the hidden depths of the biosphere. *Trends Microbiol.* **13**, 401.

Cacchio, P., Contento, R., Ercole, C., Cappuccio, G., Martinez, M. P., and Lepidi, A. (2004). Involvement of microorganisms in the formation of carbonate speleothems in the Cervo Cave (L'Aquila-Italy). *Geomicrobiol. J.* **21**, 497–509.

Cameron, D. G., Martin, A., and Mantsch, H. H. (1983). Membrane isolation alters the gel to liquid-crystal transition of Acholeplasma-laidlawii-B. *Science* **219**, 180–182.

Campen, R. K., Sowers, T., and Alley, R. B. (2003). Evidence of microbial consortia metabolizing within a low-latitude mountain glacier. *Geology* **31**, 231–234.

Carr, G. L. (1999). High-resolution microspectroscopy and sub-nanosecond time-resolved spectroscopy with the synchrotron infrared source. *Vib. Spectrosc.* **19**, 53–60.

Carr, G. L. (2001). Resolution limits for infrared microspectroscopy explored with synchrotron radiation. *Rev. Sci. Instrum.* **72**(3), 1613–1619.

Carr, G. L., Reffner, J. A., and Williams, G. P. (1995). Performance of an infrared microspectrometer at the NSLS. *Rev. Sci. Instrum.* **66**, 1490–1492.

Carrasco-Flores, E. A., Clavijo, R. E., Campos-Vallette, M. M., and Aroca, R. F. (2004). Vibrational spectra and surface-enhanced vibrational spectra of 1-nitropyrene. *Appl. Spectrosc.* **58**, 555–561.

Carrasco-Flores, E. A., Clavijo, R. E., Campos-Vallette, M. M., and Aroca, R. F. (2005). Vibrational and surface-enhanced vibrational spectra of 6-nitrochrysene. *Spectrochim. Acta A* **61**, 509–514.

Centeno, J. A., and Specht, C. S. (1992). Infrared microspectroscopy of extracellular-matrix proteins in macular and stroma scar of the cornea. *FASEB J.* **6**, A478.

Centeno, J. A., Kalasinsky, V. F., Johnson, F. B., Vinh, T. N., and Oleary, T. J. (1992). Fourier-transform infrared microscopic identification of foreign materials in tissue-sections. *Lab. Invest.* **66**, 123–131.

Chauhan, H. P. S., Shaik, N. M., and Kori, K. (2004). Synthesis and characterization of some toluene-3,4-dithiolatobismuth(III) alkyl dithiocarbonates. *Synth. React. Inorg. Me.* **34**, 323–333.

Cheah, S. F., Kraemer, S. M., Cervini-Silva, J., and Sposito, G. (2003). Steady-state dissolution kinetics of goethite in the presence of desferrioxamine B and oxalate ligands: Implications for the microbial acquisition of iron. *Chem. Geol.* **198**, 63–75.

Chinthamreddy, S., and Reddy, K. R. (1999). Oxidation and mobility of trivalent chromium in manganese-enriched clays during electrokinetic remediation. *J. Soil Contam.* **8**, 197–216.

Choo-Smith, L. P., Maquelin, K., van Vreeswijk, T., Bruining, H. A., Puppels, G. J., Thi, N. A. G., Kirschner, C., Naumann, D., Ami, D., Villa, A. M., Orsini, F., Doglia, S. M., *et al.* (2001). Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy. *Appl. Environ. Microbiol.* **67**, 1461–1469.

Coates, V. J., Offner, A., and Siegler, E. H. (1953). Design and performance of an infrared microscope attachment. *J. Opt. Soc. Am.* **43**, 984–989.

Codd, R., and Lay, P. A. (1999). Competition between 1,2-diol and 2-hydroxy acid coordination in Cr(V)-quinic acid complexes: Implications for stabilization of Cr(V) intermediates of relevance to Cr(VI)-induced carcinogenesis. *J. Am. Chem. Soc.* **121**, 7864–7876.

Codd, R., and Lay, P. A. (2001). Chromium(V)-sialic (neuraminic) acid species are formed from mixtures of chromium(VI) and saliva. *J. Am. Chem. Soc.* **123**, 11799–11800.

Codd, R., Lay, P. A., and Levina, A. (1997). Stability and ligand exchange reactions of chromium(IV) carboxylato complexes in aqueous solutions. *Inorg. Chem.* **36**, 5440–5448.

Collins, A. H. (1991). Thermal infrared-spectra and images of altered volcanic-rocks in the Virginia Range, Nevada. *Int. J. Remote Sens.* **12**, 1559–1574.

Cooley, J. W., and Tukey, J. W. (1965). An algorithm for machine calculation of complex Fourier series. *Math. Comput.* **19**, 297–301.

Cooper, D. C., Neal, A. L., Kukkadapu, R. K., Brewe, D., Coby, A., and Picardal, F. W. (2005). Effects of sediment iron mineral composition on microbially mediated changes in divalent metal speciation: Importance of ferrihydrite. *Geochim. Cosmochim. Acta* **69**, 1739–1754.

da Silva, M., Cerniglia, C. E., Pothuluri, J. V., Canhos, V. P., and Esposito, E. (2003). Screening filamentous fungi isolated from estuarine sediments for the ability to oxidize polycyclic aromatic hydrocarbons. *World J. Microbiol. Biot.* **19**, 399–405.

Daoud, C., Joly, P., Angiboust, J. F., and Manfait, M. (1988). Fourier-transform infrared microspectroscopy of isolated cells. *Ann. Biol. Clin.-Paris* **46**, 577.

De Stasio, G., Gilbert, B., Frazer, B. H., Nealson, K. H., Conrad, P. G., Livi, V., Labrenz, M., and Banfield, J. F. (2001). The multidisciplinarity of spectromicroscopy: From geomicrobiology to archaeology. *J. Electron Spectrosc.* **114**, 997–1003.

Dees, P. M., and Ghiorse, W. C. (2001). Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. *FEMS Microbiol. Ecol.* **35**, 207–216.

Delineau, T., Allard, T., Muller, J. P., Barres, O., Yvon, J., and Cases, J. M. (1994). FTIR reflectance vs EPR studies of structural iron in kaolinites. *Clay Clay Miner.* **42**, 308–320.

Dillon, C. T., Lay, P. A., Cholewa, M., Legge, G. J. F., Bonin, A. M., Collins, T. J., Kostka, K. L., and SheaMcCarthy, G. (1997). Microprobe X-ray absorption spectroscopic determination of the oxidation state of intracellular chromium following exposure of V79 Chinese hamster lung cells to genotoxic chromium complexes. *Chem. Res. Toxicol.* **10**, 533–535.

Dokken, K. M., Davis, L. C., Erickson, L. E., Castro-Diaz, S., and Marinkovic, N. S. (2005a). Synchrotron fourier transform infrared micro spectroscopy: A new tool to monitor the fate of organic contaminants in plants. *Microchem. J.* **81**, 86–91.

Dokken, K. M., Davis, L. C., and Marinkovic, N. S. (2005b). Use of infrared microspectroscopy in plant growth and development. *Appl. Spectrosc. Rev.* **40**, 301–326.

Dokken, K. M., Davis, L. C., and Marinkovic, N. S. (2005c). Using SR-IMS to study the fate and transport of organic contaminants in plants. *Spectroscopy* **20**, 14–18.

Douglas, S., and Douglas, D. D. (2001). Structural and geomicrobiological characteristics of a microbial community from a cold sulfide spring. *Geomicrobiol J.* **18**, 401–422.

Dumas, P., and Miller, L. (2003). The use of synchrotron infrared microspectroscopy in biological and biomedical investigations. *Vib. Spectrosc.* **32**, 3–21.

Dumas, P., Jamin, N., Teillaud, J. L., Miller, L. M., and Beccard, B. (2004). Imaging capabilities of synchrotron infrared microspectroscopy. *Faraday Discuss.* **126**, 289–302.

Edwards, K. J., Bond, P. L., Druschel, G. K., McGuire, M. M., Hamers, R. J., and Banfield, J. F. (2000). Geochemical and biological aspects of sulfide mineral dissolution: Lessons from Iron Mountain, California. *Chem. Geol.* **169**, 383–397.

Edwards, K. J., Hu, B., Hamers, R. J., and Banfield, J. F. (2001). A new look at microbial leaching patterns on sulfide minerals. *FEMs Microbiol. Ecol.* **34**, 197–206.

Edwards, K. J., Bach, W., and Rogers, D. R. (2003). Geomicrobiology of the ocean crust: A role for chemoautotrophic Fe-bacteria. *Biol. Bull.* **204**, 180–185.

Edwards, K. J., Bach, W., and McCollom, T. M. (2005). Geomicrobiology in oceanography: Microbe-mineral interactions at and below the seafloor. *Trends Microbiol.* **13**, 449–456.

Ehrlich, H. L. (1994). Special issue: Breakthroughs in karst geomicrobiology and redox geochemistry—foreword. *Geomicrobiol. J.* **12**, 135.

Ehrlich, H. L. (1998). Geomicrobiology: Its significance for geology. *Earth-sci. Rev.* **45**, 45–60.

Ehrlich, H. L. (2000). Geomicrobiology. *Geotimes* **45**, 34–37.

Emerson, D., and Ghiorse, W. C. (1993). Ultrastructure and chemical-composition of the sheath of *Leptothrix discophora* Sp-6. *J. Bacteriol.* **175**, 7808–7818.

Engel, A. S., Stern, L. A., and Bennett, P. C. (2004). Microbial contributions to cave formation: New insights into sulfuric acid speleogenesis. *Geology* **32**, 369–372.

Eyring, E. M., and Wadsworth, M. E. (1956). Differential infrared spectra of adsorbed monolayers-normal-hexanethiol on Zn minerals. *T. Am. I. Min. Met. Eng.* **205**, 531–535.

Facciotti, M. T., Rouhani, S., Burkard, F. T., Betancourt, F. M., Downing, K. H., Rose, R. B., McDermott, G., and Glaeser, R. M. (2001). Structure of an early intermediate in the M-state phase of the bacteriorhodopsin photocycle. *Biophys. J.* **81**, 3442–3455.

Farmer, V. C. (Ed.) (1974). "The Infrared Spectra of Minerals." Mineralogical Society, London.

Fein, J. B., Fowle, D. A., Cahill, J., Kemner, K., Boyanov, M., and Bunker, B. (2002). Nonmetabolic reduction of Cr(VI) by bacterial surfaces under nutrient-absent conditions. *Geomicrobiol. J.* **19**, 369–382.

Foriel, J., Philippot, P., Susini, J., Dumas, P., Somogyi, A., Salome, M., Khodja, H., Menez, M., Fouquet, Y., Moreira, D., and Lopez-Garcia, P. (2004). High-resolution imaging of sulfur oxidation states, trace elements, and organic molecules distribution in individual microfossils and contemporary microbial filaments. *Geochim. Cosmochim. Acta* **68**, 1561–1569.

Francis, A. J., Dodge, C. J., Gillow, J. B., and Papenguth, H. W. (2000). Biotransformation of uranium compounds in high ionic strength brine by a halophilic bacterium under denitrifying conditions. *Environ. Sci. Technol.* **34**, 2311–2317.

Francis, A. J., Gillow, J. B., Dodge, C. J., Harris, R., Beveridge, T. J., and Papenguth, H. W. (2004). Uranium association with halophilic and non-halophilic bacteria and archaea. *Radiochim. Acta* **92**, 481–488.

Fredrickson, J. K., Zachara, J. M., Balkwill, D. L., Kennedy, D., Li, S. M. W., Kostandarithes, H. M., Daly, M. J., Romine, M. F., and Brockman, F. J. (2004). Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford site, Washington State. *Appl. Environ. Microbiol.* **70**, 4230–4241.

Furukawa, K. (2000). Biochemical and genetic bases of microbial degradation of polychlorinated biphenyls (PCBs). *J. Gen. Appl. Microbiol.* **46**, 283–296.

Furukawa, K. (2003). "Super bugs" for bioremediation. *Trends Biotechnol.* **21**, 187–190.

Furukawa, K., Hirose, J., Suyama, A., Zaiki, T., and Hayashida, S. (1993). Gene components responsible for discrete substrate-specificity in the metabolism of biphenyl (Bph operon) and toluene (Tod operon). *J. Bacteriol.* **175**, 5224–5232.

Furukawa, K., Suenaga, H., and Goto, M. (2004). Biphenyl dioxygenases: Functional versatilities and directed evolution. *J. Bacteriol.* **186**, 5189–5196.

Gez, S., Luxenhofer, R., Levina, A., Codd, R., and Lay, P. A. (2005). Chromium(V) complexes of hydroxamic acids: Formation, structures, and reactivities. *Inorg. Chem.* **44**, 2934–2943.

Ghiorse, W. C., and Chapnick, S. D. (1983). Metal-depositing bacteria and the distribution of manganese and iron in swamp waters. *Ecol. Bull.* **35**, 367–376.

Ghiorse, W. C., and Hirsch, P. (1979). Ultrastructural-study of iron and manganese deposition associated with extracellular polymers of pedomicrobium-like budding bacteria. *Arch. Microbiol.* **123**, 213–226.

Ghosh, U., Talley, J. W., and Luthy, R. G. (2001). Particle-scale investigation of PAH desorption kinetics and thermodynamics from sediment. *Environ. Sci. Technol.* **35**, 3468–3475.

Goodhue, L. D., Hamilton, S., and Southam, G. (2005). The geomicrobiology of surficial geochemical anomalies. *Geochim. Cosmochim. Acta* **69**, A367.

Gordon, G. E., and Sturchio, N. C. (2002). An overview of synchrotron radiation applications to low temperature geochemistry and environmental science. *In* "Reviews in Mineralogy & Geochemistry" (P. A. Fenter, M. L. Rivers, N. C. Sturchio, and S. R. Sutton, Eds.), The Mineralogical Society of America, Vol. 69, pp. 1–116, Washington DC.

Gore, R. C. (1949). Infrared spectrometry of small samples with the reflecting microscope. *Science* **110**, 710–711.

Griffith, W. P., Lewis, J., and Wilkinson, G. (1959). Infrared spectra of transition metal-nitric oxide complexes.4. The pentacyanonitrosyl-complexes of chromium and molybdenum. *J. Am. Chem. Soc.* (MAR) 872–875.

Guilhaumou, N., Dumas, P., Carr, G. L., and Williams, G. P. (1998). Synchrotron infrared microspectrometry applied to petrography in micrometer-scale range: Fluid chemical analysis and mapping. *Appl. Spectrosc.* **52**(8), 1029–1034.

Ha, Y. W., Stack, R. J., Hespell, R. B., Gordon, S. H., and Bothast, R. J. (1991). Some chemical and physical-properties of extracellular polysaccharides produced by butyrivibrio-fibrisolvens strains. *Appl. Environ. Microbiol.* **57**, 2016–2020.

Hale, D. D., Rogers, J. E., and Wiegel, J. (1990a). Reductive dechlorination of dichlorophenols by nonadapted and adapted microbial communities in pond sediments. *Microbial Ecol.* **20**, 185–196.

Hale, D. D., Rogers, J. E., and Wiegel, J. W. (1990b). Reductive dechlorination of dichlorophenols in anaerobic pond sediments. *Abst. Pap. Am. Chem. S.* **199**, 137.

Haugland, R. P. (2002). ''Handbook of Fluorescent Probes and Research Products,'' Molecular Probes. http://probes.invitrogen.com/

Hawkins, N. J., Mattraw, H. C., Sabol, W. W., and Carpenter, D. R. (1955). Spectroscopy of gaseous carbonyls.1. Infrared spectra and thermodynamic properties of chromium and molybdenum hexacarbonyls. *J. Chem. Phys.* **23**, 2422–2427.

Helm, D., and Naumann, D. (1995). Identification of some bacterial-cell components by FT-IR spectroscopy. *FEMS Microbiol. Lett.* **126**, 75–79.

Helm, D., Labischinski, H., and Naumann, D. (1991a). Elaboration of a procedure for identification of bacteria using Fourier-transform IR spectral libraries—a stepwise correlation approach. *J. Microbiol. Meth.* **14**, 127–142.

Helm, D., Labischinski, H., Schallehn, G., and Naumann, D. (1991b). Classification and identification of bacteria by Fourier-transform infrared-spectroscopy. *J. Gen. Microbiol.* **137**, 69–79.

Hines, M. E., Orem, W. H., Lyons, W. B., and Jones, G. E. (1982). Microbial activity and bioturbation induced oscillations in pore water chemistry of estuarine sediments in spring. *Nature* **299**, 433–435.

Hirschmugl, C. J. (2002a). Applications of storage ring infrared spectromicroscopy and reflection-absorption spectroscopy to geochemistry and environmental science. *Rev. Mineral. Geochem.* **49**, 317–339.

Hirschmugl, C. J. (2002b). Frontiers in infrared spectroscopy at surfaces and interfaces. *Surf. Sci.* **500**, 577–604.

Holman, H. N., Martin, M. C., Blakely, E. A., Bjornstad, K., and McKinney, W. R. (2000a). IR spectroscopic characteristics of cell cycle and cell death probed by synchrotron radiation based fourier transform IR spectromicroscopy. *Biopolymers* **57**, 329–335.

Holman, H. Y. N. (2004). Tracking microbial interactions with oxyanions by synchrotron infrared spectromicroscopy. *Abst. Pap. Am. Chem. S.* **228**, U619.

Holman, H. Y. N., Perry, D. L., and Hunter-Cevera, J. C. (1998). Surface-enhanced infrared absorption-reflectance (SEIRA) microspectroscopy for bacteria localization on geologic material surfaces. *J. Microbiol. Meth.* **34**, 59–71.

Holman, H. Y. N., Perry, D. L., Martin, M. C., Lamble, G. M., McKinney, W. R., and Hunter-Cevera, J. C. (1999). Real-time characterization of biogeochemical reduction of Cr(VI) on basalt surfaces by SR-FTIR imaging. *Geomicrobiol. J.* **16**, 307–324.

Holman, H. Y. N., Goth-Goldstein, R., Martin, M. C., Russell, M. L., and McKinney, W. R. (2000b). Low-dose responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin in single living human cells measured by synchrotron infrared spectromicroscopy. *Environ. Sci. Technol.* **34**, 2513–2517.

Holman, H. Y. N., Bjornstad, K. A., McNamara, M. P., Martin, M. C., McKinney, W. R., and Blakely, E. A. (2002a). Synchrotron infrared spectromicroscopy as a novel bioanalytical microprobe for individual living cells: Cytotoxicity considerations. *J. Biomed. Opt.* **7**, 417–424.

Holman, H. Y. N., Nieman, K., Sorensen, D. L., Miller, C. D., Martin, M. C., Borch, T., McKinney, W. R., and Sims, R. C. (2002b). Catalysis of PAH biodegradation by humic acid shown in synchrotron infrared studies. *Environ. Sci. Technol.* **36**, 1276–1280.

Holman, H. Y. N., Martin, M. C., and McKinney, W. R. (2003). Tracking chemical changes in a live cell: Biomedical applications of SR-FTIR spectromicroscopy. *Spectrosc.—Int. J.* **17**, 139–159.

Holman, H. Y. N., Lin, Z., Asatiani, N. V., Kalabegishvili, T., Sapojnikova, N. A., Martin, M. C., McKinney, W. R., and Tsibakhashvili, N. Y. (2004). Role of the survival strategy of arthrobacters in the geochemical cycling of chromium—a spectroscopy and microscopy study. *Abst. Pap. Am. Chem. S.* **227**, U1221.

Hudgins, D. M., and Sandford, S. A. (1998a). Infrared spectroscopy of matrix isolated polycyclic aromatic hydrocarbons. 1. PAHs containing two to four rings. *J. Phys. Chem. A* **102**, 329–343.

Hudgins, D. M., and Sandford, S. A. (1998b). Infrared spectroscopy of matrix isolated polycyclic aromatic hydrocarbons. 2. PAHs containing five or more rings. *J. Phys. Chem. A* **102**, 344–352.

Hudgins, D. M., and Sandford, S. A. (1998c). Infrared spectroscopy of matrix isolated polycyclic aromatic hydrocarbons. 3. Fluoranthene and the benzofluoranthenes. *J. Phys. Chem. A* **102**, 353–360.

Hudgins, D. M., Bauschlicher, C. W., Allamandola, L. J., and Fetzer, J. C. (2000). Infrared spectroscopy of matrix-isolated polycyclic aromatic hydrocarbon ions. 5. PAHs incorporating a cyclopentadienyl ring. *J. Phys. Chem. A* **104**, 3655–3669.

Humphrey, R. E. (1961). The infrared spectra of aromatic chromium tricarbonyls. *Spectrochim. Acta* **17**, 93–100.

IARC (1990). ''Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.'' International Agency for Research on Cancer, Lyon, France.

Jamin, N., Dumas, P., Moncuit, J., Fridman, W. H., Teillaud, J. L., Carr, G. L., and Williams, G. P. (1998). Highly resolved chemical imaging of living cells by using synchrotron infrared microspectrometry. *Proc. Natl. Acad. Sci. USA* **95**, 4837–4840.

Janni, J., Gilbert, B. D., Field, R. W., and Steinfeld, J. I. (1997). Infrared absorption of explosive molecule vapors. *Spectrochim. Acta A* **53**, 1375–1381.

Jensen, J. O. (2004a). Vibrational frequencies and structural determination of arsenous tricyanide. *J. Mol. Struc.–Theochem.* **678**, 195–199.

Jensen, J. O. (2004b). Vibrational frequencies and structural determination of phosphorous tricyanide. *Spectrochim. Acta A* **60**, 2537–2540.

Jensen, J. O., and Jensen, J. L. (2004). Vibrational frequencies and structural determination of trimethylarsine oxide. *Spectrochim. Acta A* **60**, 3065–3070.

Joeckel, R. M., and Clement, B. J. A. (2005). Soils, surficial geology, and geomicrobiology of saline-sodic wetlands, North Platte River Valley, Nebraska, USA. *Catena* **61**, 63–101.

Jones, R. A., Koval, S. F., and Nesbitt, H. W. (2003). Surface alteration of arsenopyrite (FeAsS) by *Thiobacillus ferrooxidans*. *Geochim. Cosmochim. Acta* **67**, 955–965.

Jurgensen, A., Widmeyer, J. R., Gordon, R. A., Bendell-Young, L. I., Moore, M. M., and Crozier, E. D. (2004). The structure of the manganese oxide on the sheath of the bacterium *Leptothrix discophora:* An XAFS study. *Am. Mineral.* **89**, 1110–1118.

Kahng, L. S., and Shapiro, L. (2003). Polar localization of replicon origins in the multipartite genomes of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*. *J. Bacteriol.* **185**, 3384–3391.

Kalabegishvili, T. L., Tsibakhashvili, N. Y., and Holman, H. Y. N. (2003). Electron spin resonance study of chromium(V) formation and decomposition by basalt-inhabiting bacteria. *Environ. Sci. Technol.* **37**, 4678–4684.

Kalinowski, B. E., Liermann, L. J., Brantley, S. L., Barnes, A., and Pantano, C. G. (2000). X-ray photoelectron evidence for bacteria-enhanced dissolution of hornblende. *Geochim. Cosmochim. Acta* **64**, 1331–1343.

Kansiz, M., Heraud, P., Wood, B., Burden, F., Beardall, J., and McNaughton, D. (1999). Fourier transform infrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains. *Phytochemistry* **52**, 407–417.

Keller, W. D., Spotts, J. H., and Biggs, D. L. (1952). Infrared spectra of some rock-forming minerals. *Am. J. Sci.* **250**, 453.

Khijniak, T. V., Slobodkin, A. I., Coker, V., Renshaw, J. C., Livens, F. R., Bonch-Osmolovskaya, E. A., Birkeland, N. K., Medvedeva-Lyalikova, N. N., and Lloyd, J. R. (2005). Reduction of uranium(VI) phosphate during growth of the thermophilic bacterium *Thermoterrabacterium ferrireducens*. *Appl. Environ. Microbiol.* **71**, 6423–6426.

Kilkenny, D. M., Rocheleau, J. V., Piston, D. W., and Miller, G. G. (2002). c-Src expression decreases sensitivity to fibroblast growth factor (FGF)-1-induced chemotaxis and chemokinesis. *Mol. Biol. Cell* **13**, 329a.

Kim, Y. H., Pak, K., Pothuluri, J. V., and Cerniglia, C. E. (2004). Mineralization of erythromycin A in aquaculture sediments. *FEMS Microbiol. Lett.* **234**, 169–175.

Kirschner, C., Maquelin, K., Pina, P., Thi, N. A. N., Choo-Smith, L. P., Sockalingum, G. D., Sandt, C., Ami, D., Orsini, F., Doglia, S. M., Allouch, P., Mainfait, M., *et al.* (2001). Classification and identification of enterococci: A comparative phenotypic, genotypic, and vibrational spectroscopic study. *J. Clin. Microbiol.* **39**, 1763–1770.

Koenigsberg, S. S., Hazen, T. C., and Peacock, A. D. (2005). Environmental biotechnology: A bioremediation perspective. *Remediation Journal* **15**, 5–25.

Kretzschmar, R., Robarge, W. P., and Weed, S. B. (1993). Flocculation of kaolinitic soil clays: Effects of humic substances and iron-oxides. *Soil Sci. Soc. Am. J.* **57**, 1277–1283.

Krumholz, L. R., McKinley, J. P., Ulrich, F. A., and Suflita, J. M. (1997). Confined subsurface microbial communities in Cretaceous rock. *Nature* **386**, 64–66.

Kumamaru, T., Suenaga, H., Mitsuoka, M., Watanabe, T., and Furukawa, K. (1998). Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. *Nat. Biotechnol.* **16**, 663–666.

Labischinski, H., Naumann, D., Schultz, C., Kusumoto, S., Shiba, T., Rietschel, E. T., and Giesbrecht, P. (1989). Comparative X-Ray and Fourier-transform-infrared investigations of conformational properties of bacterial and synthetic lipid-a of *Escherichia coli* and *Salmonella minnesota* as well as partial structures and analogs thereof. *Eur. J. Biochem.* **179**, 659–665.

Lack, J. G., Chaudhuri, S. K., Kelly, S. D., Kemner, K. M., O'Connor, S. M., and Coates, J. D. (2002). Immobilization of radionuclides and heavy metals through anaerobic bio-oxidation of Fe(II). *Appl. Environ. Microbiol.* **68**, 2704–2710.

Lajtha, K., and Schlesinger, W. H. (1988). The biogeochemistry of phosphorus cycling and phosphorus availability along a desert soil chronosequence. *Ecology* **69**, 24–39.

Lang, P. L., and Sang, S. C. (1998). The *in situ* infrared microspectroscopy of bacterial colonies on agar plates. *Cell. Mol. Biol.* **44**, 231–238.

Latouche, G., Cerovic, Z. G., Montagnini, F., and Moya, I. (2000). Light-induced changes of NADPH fluorescence in isolated chloroplasts: A spectral and fluorescence lifetime study. *BBA–Bioenergetics* **1460**, 311–329.

Leveille, R. J., Fyfe, W. S., and Longstaffe, F. J. (2000). Geomicrobiology of carbonate-silicate microbialites from Hawaiian basaltic sea caves. *Chem. Geol.* **169**, 339–355.

Levina, A., Barr-David, G., Codd, R., Lay, P. A., Dixon, N. E., Hammershoi, A., and Hendry, P. (1999). *In vitro* plasmid DNA cleavage by chromium(V) and -(IV) 2-hydroxycarboxylato complexes. *Chem. Res. Toxicol.* **12**, 371–381.

Levina, A., Codd, R., Dillon, C. T., and Lay, P. A. (2003). Chromium in biology: Toxicology and nutritional aspects. *Prog. Inorg. Chem.* **51**, 145–250.

Levina, A., Codd, R., Foran, G. J., Hambley, T. W., Maschmeyer, T., Masters, A. F., and Lay, P. A. (2004). X-ray absorption spectroscopic studies of chromium(V/IV/III)2-ethyl-2-hydroxybutanoato(2-/1-) complexes. *Inorg. Chem.* **43**, 1046–1055.

Levinson, E., Lerch, P., and Martin, M. C. (2006). Infrared imaging: Synchrotrons vs. arrays, resolution vs. speed. *Infrared Phys. Techn.* (in press).

Li, J. F., Fuller, S., Cattle, J., Way, C. P., and Hibbert, D. B. (2004). Matching fluorescence spectra of oil spills with spectra from suspect sources. *Anal. Chim. Acta* **514**, 51–56.

Li, Z. R., Shokes, J. E., Kounosu, A., Imai, T., Iwasaki, T., and Scott, R. A. (2003). X-ray absorption spectroscopic analysis of reductive [2Fe-2S] cluster degradation in hyperthermophilic archaeal succinate: Caldariellaquinone oxidoreductase subunits. *Biochemistry* **42**, 15003–15008.

Lieberman, R. L., Shrestha, D. B., Doan, P. E., Hoffman, B. M., Stemmler, T. L., and Rosenzweig, A. C. (2003). Purified particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a dimer with both mononuclear copper and a copper-containing cluster. *Proc. Natl. Acad. Sci. USA* **100**, 3820–3825.

Lijour, Y., Gentric, E., Deslandes, E., and Guezennec, J. (1994). Estimation of the sulfate content of hydrothermal vent bacterial polysaccharides by Fourier-transform infrared-spectroscopy. *Anal. Biochem.* **220**, 244–248.

Lovley, D. R., and Phillips, E. J. P. (1992). Reduction of uranium by *Desulfovibrio desulfuricans*. *Appl. Environ. Microbiol.* **58**, 850–856.

Lovley, D. R., and Woodward, J. C. (1996). Mechanisms for chelator stimulation of microbial Fe(III)-oxide reduction. *Chem. Geol.* **132**, 19–24.

Lovley, D. R., Roden, E. E., Phillips, E. J. P., and Woodward, J. C. (1993a). Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Mar. Geol.* **113**, 41–53.

Lovley, D. R., Widman, P. K., Woodward, J. C., and Phillips, E. J. P. (1993b). Reduction of uranium by cytochrome-C(3) of *Desulfovibrio vulgaris*. *Appl. Environ. Microbiol.* **59**, 3572–3576.

Lu, R., Goncharov, A., Mao, H. K., and Hemley, R. (1999). Synchrotron infrared microspectroscopy: Applications to hydrous minerals. *In* "Synchrotron X-ray Methods in Clay Sciences" (D. G. Schulze, J. W. Stucki, and P. M. Bertsch, Eds.), pp. 164–182. The Clay Mineral Society, Boulder.

Ludwig, C., Dolny, M., and Gotze, H. J. (2000). Fourier transform Raman and infrared spectra and normal coordinate analysis of organo-arsenic(III), -antimony(III) and -bismuth(III) thiolates. *Spectrochim. Acta A* **56**, 547–555.

Luys, M. J., Deroy, G., Vansant, E. F., and Adams, F. (1982). Characteristics of asbestos minerals: Structural aspects and infrared-spectra. *J. Chem. Soc.–Faraday T. I* **78**, 3561–3571.

Lyons, W. B., Gaudette, H. E., and Armstrong, P. B. (1979). Evidence for organically associated iron in nearshore pore fluids. *Nature* **282**, 202–203.

Macalady, J., and Banfield, J. F. (2003). Molecular geomicrobiology: Genes and geochemical cycling. *Earth Planet. Sc. Lett.* **209**, 1–17.

Maquelin, K., Kirschner, C., Choo-Smith, L. P., van den Braak, N., Endtz, H. P., Naumann, D., and Puppels, G. J. (2002). Identification of medically relevant microorganisms by vibrational spectroscopy (Review). *J. Microbiol. Meth.* **51**, 255–271.

Maquelin, K., Kirschner, C., Choo-Smith, L. P., Ngo-Thi, N. A., van Vreeswijk, T., Stammler, M., Endtz, H. P., Bruining, H. A., Naumann, D., and Puppels, G. J. (2003). Prospective study of the performance of vibrational spectroscopies for rapid identification of bacterial and fungal pathogens recovered from blood cultures. *J. Clin. Microbiol.* **41**, 324–329.

Martin, M. C., Tsvetkova, N. M., Crowe, J. H., and McKinney, W. R. (2001). Negligible sample heating from synchrotron infrared beam. *Appl. Spectrosc.* **55**, 111–113.

Mattioda, A. L., Hudgins, D. M., Bauschlicher, C., and Allamandola, L. J. (2002). Infrared spectra of matrix-isolated polycyclic aromatic hydrocarbons and their ions: PAHs incorporating the peropyrene structure. *Abst. Pap. Am. Chem. S.* **224**, U323.

Maurice, P. A., Vierkorn, M. A., Hersman, L. E., and Fulghum, J. E. (2001). Dissolution of well and poorly ordered kaolinites by an aerobic bacterium. *Chem. Geol.* **180**, 81–97.

McCann, M. C., Hammouri, M., Wilson, R., Belton, P., and Roberts, K. (1992). Fourier-transform infrared microspectroscopy is a new way to look at plant-cell walls. *Plant Physiol.* **100**, 1940–1947.

McKinney, W. R., Martin, M. C., Chin, M., Portman, G., Melczer, M. E., and Watson, J. A. (2000). 4 Axis implementation of the active feedback mirror system for the IR beamline 1.4.3. *ALS Compendium*. http://infrared.als.lbl.gov/

McMahon, P. B., and Chapelle, F. H. (1991). Microbial-production of organic-acids in aquitard sediments and its role in aquifer geochemistry. *Nature* **349**, 233–235.

Merroun, M. L., Raff, J., Rossberg, A., Hennig, C., Reich, T., and Selenska-Pobell, S. (2005). Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. *Appl. Environ. Microbiol.* **71**, 5532–5543.

Mielczarski, J. A., Cases, J. M., Bouquet, E., Barres, O., and Delon, J. F. (1993). Nature and structure of adsorption layer on apatite contacted with oleate solution.1. Adsorption and Fourier-transform infrared reflection studies. *Langmuir* **9**, 2370–2382.

Miller, D. N., Yavitt, J. B., Madsen, E. L., and Ghiorse, W. C. (2004). Methanotrophic activity, abundance, and diversity in forested swamp pools: Spatiotemporal dynamics and influences on methane fluxes. *Geomicrobiol. J.* **21**, 257–271.

Miller, L. M., Carlson, C. S., Carr, G. L., and Chance, M. R. (1998). A method for examining the chemical basis for bone disease: Synchrotron infrared microspectroscopy. *Cell. Mol. Biol.* **44**, 117–127.

Miller, L. M., Dumas, P., Jamin, N., Teillaud, J. L., Bantignies, J. L., and Carr, G. L. (2000). Applications of synchrotron infrared microspectroscopy to the study of biological cells and tissues. *Inst. Phys. Conf. Ser.* **165**, 75–76.

Miller, L. M., Dumas, P., Jamin, N., Teillaud, J. L., Miklossy, J., and Forro, L. (2002). Combining IR spectroscopy with fluorescence imaging in a single microscope: Biomedical applications using a synchrotron infrared source (invited). *Rev. Sci. Instrum.* **73**, 1357–1360.

Misawa, N., Shindo, K., Takahashi, H., Suenaga, H., Iguchi, K., Okazaki, H., Harayama, S., and Furukawa, K. (2002). Hydroxylation of various molecules including heterocyclic aromatics using recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes. *Tetrahedron* **58**, 9605–9612.

Naumann, D., Barnickel, G., Bradaczek, H., Labischinski, H., and Giesbrecht, P. (1982). Infrared-spectroscopy, a tool for probing bacterial peptidoglycan: Potentialities of infrared-spectroscopy for cell-wall analytical studies and rejection of models based on crystalline chitin. *Eur. J. Biochem.* **125**, 505–515.

Naumann, D., Fijala, V., Labischinski, H., and Giesbrecht, P. (1988). The rapid differentiation and identification of pathogenic bacteria using Fourier-transform infrared spectroscopic and multivariate statistical-analysis. *J. Mol. Struct.* **174**, 165–170.

Naumann, D., Schultz, C. P., and Helm, D. (1996). What can infrared spectroscopy tell us about the structure and composition of intact bacterial cells? *In* "Infrared Spectroscopy of Biomolecules" (H. H. Mantsch and D. Chapman, Eds.), pp. 279–310. Wiley-Liss, New York.

Neal, A. L., Amonette, J. E., Peyton, B. M., and Geesey, G. G. (2004a). Uranium complexes formed at hematite surfaces colonized by sulfate-reducing bacteria. *Environ. Sci. Technol.* **38**, 3019–3027.

Neal, A. L., Clough, L. K., Perkins, T. D., Little, B. J., and Magnuson, T. S. (2004b). *In situ* measurement of Fe(III) reduction activity of Geobacter pelophilus by simultaneous *in situ* RT-PCR and XPS analysis. *FEMS Microbiol. Ecol.* **49**, 163–169.

Nesterova, M., Moreau, J., and Banfield, J. F. (2003). Model biomimetic studies of templated growth and assembly of nanocrystalline FeOOH. *Geochim. Cosmochim. Acta* **67**, 1177–1187.

Newo, A. N. S., Pshenichnikova, A. B., Skladnev, D. A., and Shvets, V. I. (2004). Deuterium oxide as a stress factor for the methylotrophic bacterium Methylophilus sp. B-7741. *Microbiology* **73**, 139–142.

Ngo-Thi, N. A., Kirschner, C., and Naumann, D. (2003). Characterization and identification of microorganisms by FIF-IR microspectrometry. *J. Mol. Struct.* **661**, 371–380.

Nguyen, T. T., Janik, L. J., and Raupach, M. (1991). Diffuse reflectance infrared Fourier-transform (drift) spectroscopy in soil studies. *Aust. J. Soil Res.* **29**, 49–67.

Orser, C. S., Foong, F. C. F., Capaldi, R., Nalezny, J., Mackay, W., Benjamin, M., and Farr, S. B. (1995). Use of prokaryotic stress promoters as indicators of the mechanisms of chemical toxicity. *In vitro Toxicol.* **8**, 71–85.

Osborne, F. H., and Ehrlich, H. L. (1976). Oxidation of arsenite by a soil isolate of Alcaligenes. *J. Appl. Bacteriol.* **41**, 295–305.

Panak, P. J., Knopp, R., Booth, C. H., and Nitsche, H. (2002). Spectroscopic studies on the interaction of U(VI) with Bacillus sphaericus. *Radiochim. Acta* **90**, 779–783.

Panero, W. R., Benedetti, L. R., and Jeanloz, R. (2003). Transport of water into the lower mantle: Role of stishovite. *J. Geophys. Res.-Sol. Ea.* **108**(B1), 2039–2047.

Pauzat, F., and Ellinger, Y. (2001). The 3.2–3.5 mu m region revisited–II. A theoretical study of the effects of hydrogenation on some model PAHs. *Mon. Not. R. Astron. Soc.* **324**, 355–366.

Pauzat, F., and Ellinger, Y. (2002). The infrared signatures of non-regular PAHs. *Chem. Phys.* **280**, 267–282.

Peitscher, G. W. (1986). Infrared microspectroscopy of polymers. *Makromol. Chem.-M. Symp.* **5**, 75–85.

Pennisi, E. (2000). Geomicrobiology: Hardy microbe thrives at pH 0. *Science* **287**, 1731–1732.

Phillips, E. J. P., Landa, E. R., and Lovley, D. R. (1995). Remediation of uranium contaminated soils with bicarbonate extraction and microbial U(VI) reduction. *J. Ind. Microbiol.* **14**, 203–207.

Pickering, I. J., George, G. N., Yu, E. Y., Brune, D. C., Tuschak, C., Overmann, J., Beatty, J. T., and Prince, R. C. (2001). Analysis of sulfur biochemistry of sulfur bacteria using X-ray absorption spectroscopy. *Biochemistry* **40**, 8138–8145.

Piston, D. W., and Knobel, S. M. (1999). Real-time analysis of glucose metabolism by microscopy. *Trends Endocrin. Met.* **10**, 413–417.

Plesko, E. P., Scheetz, B. E., and White, W. B. (1992). Infrared vibrational characterization and synthesis of a family of hydrous alkali uranyl silicates and hydrous uranyl silicate minerals. *Am. Mineral.* **77**, 431–437.

Pothuluri, J. V., Selby, A., Evans, F. E., Freeman, J. P., and Cerniglia, C. E. (1995). Transformation of chrysene and other polycyclic aromatic hydrocarbon mixtures by the fungus *Cunninghamella-elegans*. *Can. J. Bot.-Revue Canadienne De Botanique* **73**, S1025–S1033.

Pothuluri, J. V., Doerge, D. R., Churchwell, M. I., Fu, P. P., and Cerniglia, C. E. (1998a). Fungal metabolism of nitrofluoranthenes. *J. Toxicol. Environ. Health-Part A* **53**, 153–174.

Pothuluri, J. V., Sutherland, J. B., Freeman, J. P., and Cerniglia, C. E. (1998b). Fungal biotransformation of 6-nitrochrysene. *App. Environ. Microbiol.* **64**, 3106–3109.

Pothuluri, J. V., Freeman, J. P., Fu, P. P., and Cerniglia, C. E. (1999). Biotransformation of 1-nitrobenzo[e]pyrene by the fungus *Cunninghamella elegans*. *J. Indust. Microbiol. Biotechn.* **22**, 52–57.

Povarennykh, A. S. (1978). Use of infrared-spectra for determination of minerals. *Am. Mineral.* **63**, 956–959.

Prange, A., Chauvistre, R., Modrow, H., Hormes, J., Truper, H. G., and Dahl, C. (2002a). Quantitative speciation of sulfur in bacterial sulfur globules: X-ray absorption spectroscopy reveals at least three different species of sulfur. *Microbiol.-Sgm* **148**, 267–276.

Prange, A., Dahl, C., Truper, H. G., Behnke, M., Hahn, J., Modrow, H., and Hormes, J. (2002b). Investigation of S-H bonds in biologically important compounds by sulfur K-edge X-ray absorption spectroscopy. *Eur. Phys. J. D* **20**, 589–596.

Pshenichnikova, A. B., Nevo, A. N. S., Volkova, E. V., Skladnev, D. A., and Shvets, V. I. (2004). Effect of deuteration on the activity of methanol dehydrogenase from Methylophilus sp B-7741. *Appl. Biochem. Microbiol.* **40**, 18–21.

Raab, T. K., and Vogel, J. P. (2004). Ecological and agricultural applications of synchrotron IR microscopy. *Infrared Phys. Techn.* **45**, 393–402.

Reffner, J. A., Martoglio, P. A., and Williams, G. P. (1995). Fourier-transform infrared microscopic analysis with synchrotron-radiation: The microscope optics and system performance. *Rev. Sci. Instrum.* **66**, 1298–1302.

Reffner, J. A., Carr, G. L., and Williams, G. P. (1997). Infrared microspectroscopy with synchrotron radiation. *Mikrochim. Acta* (Suppl. 14), 339–341.

Renault, P., Calero, S., Delorme, C., Drouault, S., Goupil-Feuillerat, N., Guedon, E., and Ehrlich, S. D. (1998). From genome to industrial application. *Lait* **78**, 39–52.

Renshaw, J. C., Butchins, L. J. C., Livens, F. R., May, I., Charnock, J. M., and Lloyd, J. R. (2005). Bioreduction of uranium: Environmental implications of a pentavalent intermediate. *Environ. Sci. Techn.* **39**, 5657–5660.

Revsbech, N. P., Sorensen, J., Blackburn, T. H., and Lomholt, J. P. (1980). Distribution of oxygen in marine-sediments measured with microelectrodes. *Limnol. Oceanogr.* **25**, 403–411.

Rocheleau, J. V., Edidin, M., and Piston, D. W. (2002). Monitoring the membrane rotational mobility of MHC class I protein using an intra-sequence expressed GFP construct. *Biophys. J.* **82**, 627a.

Rogers, J. E., and Hale, D. D. (1987). Anaerobic degradation of dichlorophenol isomers in fresh-water pond sediments. *Abst. Pap. Am. Chem. S.* **194**, 244.

Rossman, G. R., and Aines, R. D. (1991). The hydrous components in garnets—grossular-hydrogrossular. *Am. Mineral.* **76**, 1153–1164.

Ruiterkamp, R., Halasinski, T., Salama, F., Foing, B. H., Allamandola, L. J., Schmidt, W., and Ehrenfreund, P. (2002). Spectroscopy of large PAHs: Laboratory studies and comparison to the diffuse interstellar bands. *Astron. Astrophys.* **390**, 1153–1170.

Saita, S., and Maenosono, S. (2005). FePt nanoparticles with a narrow composition distribution synthesized via pyrolysis of iron(III) ethoxide and platinum(II) acetylacetonate. *Chem. Mater.* **17**, 3705–3710.

Salisbury, J. W., Walter, L. S., Vergo, N., and D'Aria, D. M. (1991). "Infrared (2.1–25 µm) Spectra of Minerals." The Johns Hopkins University Press, Baltimore and London.

Sanchez-Moral, S., Canaveras, J. C., Laiz, L., Saiz-Jimenez, C., Bedoya, J., and Luque, L. (2003). Biomediated precipitation of calcium carbonate metastable phases in hypogean environments: A short review. *Geomicrobiol. J.* **20**, 491–500.

Sarret, G., Avoscan, L., Carriere, M., Collins, R., Geoffroy, N., Carrot, F., Coves, J., and Gouget, B. (2005). Chemical forms of selenium in the metal-resistant bacterium *Ralstonia metallidurans* CH34 exposed to selenite and selenate. *Appl. Environ. Microbiol.* **71**, 2331–2337.

Scarvie, T., Andresen, N., Baptiste, K., Byrd, J., Chin, M., Martin, M. C., McKinney, W. R., and Steier, C. (2004). Noise reduction efforts for the ALS infrared beamlines. *Infrared Phys. Techn.* **45**, 403–408.

Schabereiter-Gurtner, C., Saiz-Jimenez, C., Pinar, G., Lubitz, W., and Rolleke, S. (2002). Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Palaeolithic paintings. *Environ. Microbiol.* **4**, 392–400.

Schultz, C., and Naumann, D. (1991). *In vivo* study of the state of order of the membranes of gram-negative bacteria by Fourier-transform infrared-spectroscopy (Ft-Ir). *FEBS Lett.* **294**, 43–46.

Schultz, C., Labischinski, H., and Naumann, D. (1987). Thermotropic phase-behavior of gram-negative bacterial lipopolysaccharides studied using Fourier-transform infrared-spectroscopy (Ft-Ir). *Z. Kristallogr.* **178**, 201–202.

Seelenbinder, J. A., and Brown, C. W. (2002). Comparison of organic self-assembled mono-layers as modified substrates for surface-enhanced infrared absorption spectroscopy. *Appl. Spectrosc.* **56**, 295–299.

Sham, T. K., and Rivers, M. L. (2002). A brief overview of synchrotron radiation. *Rev. Mineral Geochem.* **49**, 117–147.

Simon, D., Muller, R. H., Bley, T., and Babel, W. (1996). Correlation between cellular fluorescence and NAD(P)H levels in Alcaligenes eutrophus JMP 134. *Bioprocess Eng.* **14**, 57–62.

Sinclair, J. L., and Ghiorse, W. C. (1989). Distribution of aerobic-bacteria, protozoa, algae, and fungi in deep subsurface sediments. *Geomicrobiol. J.* **7**, 15–31.

Snow, E. T. (1991). A possible role for chromium(III) in genotoxicity. *Environ. Health Persp.* **92**, 75–81.

Sorensen, J., Jorgensen, B. B., and Revsbech, N. P. (1979). Comparison of oxygen, nitrate, and sulfate respiration in coastal marine-sediments. *Microb. Ecol.* **5**, 105–115.

Spilde, M. N., Northup, D. E., Boston, P. J., Schelble, R. T., Dano, K. E., Crossey, L. J., and Dahm, C. N. (2005). Geomicrobiology of cave ferromanganese deposits: A field and laboratory investigation. *Geomicrobiol. J.* **22**, 99–116.

Sreedhara, A., Susa, N., and Rao, C. P. (1997). Vanadate and chromate reduction by sacchar-ides and L-ascorbic acid: Effect of the isolated V(IV) and Cr(III) products on DNA nicking, lipid peroxidation, cytotoxicity and on enzymatic and non-enzymatic antioxidants. *Inorg. Chim. Acta* **263**, 189–194.

Stern, R. M. (1982). Biological and environmental aspects of chromium. *In* "Biological and Environmental Aspects of Chromium" (S. Langard, Ed.). Elsevier, Amsterdam.

Suenaga, H., Mitsuoka, M., Ura, Y., Watanabe, T., and Furukawa, K. (2001). Directed evolution of biphenyl dioxygenase: Emergence of enhanced degradation capacity for ben-zene, toluene, and alkylbenzenes. *J. Bacteriol.* **183**, 5441–5444.

Suenaga, H., Watanabe, T., Sato, M., Furukawa, N., and Furukawa, K. (2002). Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. *J. Bacteriol.* **184**, 3682–3688.

Suzuki, Y., and Banfield, J. F. (2004). Resistance to, and accumulation of, uranium by bacteria from a uranium-contaminated site. *Geomicrobiol. J.* **21**, 113–121.

Suzuki, Y., Kelly, S. D., Kemner, K. A., and Banfield, J. F. (2003). Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. *Appl. Environ. Microbiol.* **69**, 1337–1346.

Tebo, B. M., Ghiorse, W. C., vanWaasbergen, L. G., Siering, P. L., and Caspi, R. (1997). Bacterially mediated mineral formation: Insights into manganese(II) oxidation from molecular genetic and biochemical studies. *Rev. Mineral* **35**, 225–266.

Tebo, B. M., Bargar, J. R., Clement, B. G., Dick, G. J., Murray, K. J., Parker, D., Verity, R., and Webb, S. M. (2004). Biogenic manganese oxides: Properties and mechanisms of formation. *Annu. Rev. Earth Pl. Sc.* **32**, 287–328.

Tebo, B. M., Johnson, H. A., McCarthy, J. K., and Templeton, A. S. (2005). Geomicrobiology of manganese(II) oxidation. *Trends Microbiol.* **13**, 421–428.

Templeton, A. S., Staudigel, H., and Tebo, B. M. (2005). Diverse Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi Seamount. *Geomicrobiol. J.* **22**, 127–139.

Thanbichler, M., Viollier, P. H., and Shapiro, L. (2005). The structure and function of the bacterial chromosome. *Curr. Opin. Gen. Dev.* **15**, 153–162.

Todd, M. W., Provencal, R. A., Owano, T. G., Paldus, B. A., Kachanov, A., Vodopyanov, K. L., Hunter, M., Coy, S. L., Steinfeld, J. I., and Arnold, J. T. (2002). Application of mid-infrared cavity-ringdown ectroscopy to trace explosives vapor detection using a broadly tunable (6–8 μm) optical parametric oscillator. *Appl. Phys. B-Lasers O.* **75**, 367–376.

Toner, B., Fakra, S., Villalobos, M., Warwick, T., and Sposito, G. (2005). Spatially resolved characterization of biogenic manganese oxide production within a bacterial biofilm. *Appl. Environ. Microbiol.* **71**, 1300–1310.

Tsibakhashvili, N. Y., Asatiani, N. V., Abuladze, M. K., Birkaya, B. G., Sapojnikova, N. A., Mosulishvili, L. M., and Holman, H. Y. N. (2002a). Capillary electrophoresis of Cr(VI) reducer *Arthrobacter oxydans. Biomed. Chromatogr.* **16**, 327–331.

Tsibakhashvili, N. Y., Mosulishvili, L. M., Kalabegishvili, T. L., Pataraya, D. T., Gurielidze, M. A., Nadareishvili, G. S., and Holman, H. Y. N. (2002b). Chromate-resistant and reducing-microorganisms in georgia basalts: Their distribution and characterization. *Fresen. Environ. Bull.* **11**, 352–361.

Tsibakhashvili, N. Y., Mosulishvili, L. M., Kalabegishvili, T. L., Kirkesali, E. I., Frontasyeva, M. V., Pomyakushina, E. V., Pavlov, S. S., and Holman, H. Y. N. (2004). ENAA studies of chromium uptake by *Arthrobacter oxydans. J. Radioanal. Nucl. Chem.* **259**, 527–531.

Tsou, T. C., Lin, R. J., and Yang, J. L. (1997). Mutational spectrum induced by chromium(III) in shuttle vectors replicated in human cells: Relationship to Cr(III)-DNA interactions. *Chem. Res. Toxicol.* **10**, 962–970.

Twining, B. S., Baines, S. B., Fisher, N. S., and Landry, M. R. (2004). Cellular iron contents of plankton during the southern ocean iron experiment (SOFeX). *Deep-Sea Res.* **51**, 1827–1850.

Vandermei, H. C., Naumann, D., and Busscher, H. J. (1993). Grouping of oral streptococcal species using Fourier-transform infrared-spectroscopy in comparison with classical microbiological identification. *Arch. Oral Biol.* **38**, 1013–1019.

Vandermei, H. C., Naumann, D., and Busscher, H. J. (1996). Grouping of *Streptococcus mitis* strains grown on different growth media by Ft-Ir. *Infrared Phys. Techn.* **37**, 561–564.

Vandyk, T. K., Smulski, D. R., Reed, T. R., Belkin, S., Vollmer, A. C., and Larossa, R. A. (1995). Responses to toxicants of an *Escherichia coli* strain carrying a Uspa'–Lux genetic fusion and an *Escherichia coli* strain carrying a Grpe'–Lux fusion are similar. *Appl. Environ. Microbiol.* **61**, 4124–4127.

Vilas, F., Jarvis, K. S., and Gaffey, M. J. (1994). Iron alteration minerals in the visible and near-infrared spectra of low-Albedo asteroids. *Icarus* **109**, 274–283.

Viollier, P. H., and Shapiro, L. (2004). Spatial complexity of mechanisms controlling a bacterial cell cycle. *Curr. Opin. Microbiol.* **7**, 572–578.

Viollier, P. H., Sternheim, N., and Shapiro, L. (2002). A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. *EMBO J.* **21**, 4420–4428.

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis. Plant Cell* **14**, 2095–2106.

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *Plant J.* **40**, 968–978.

Vogt, S., Maser, J., and Jacobsen, C. (2003). Data analysis for X-ray fluorescence imaging. *J. Phys. IV* **104**, 617–622.

Voitkun, V., Zhitkovich, A., and Costa, M. (1998). Cr(III)-mediated crosslinks of glutathione or amino acids to the DNA phosphate backbone are mutagenic in human cells. *Nucleic Acids Res.* **26**, 2024–2030.

Watson, J. H. P., and Ellwood, D. C. (2003). The removal of the pertechnetate ion and actinides from radioactive waste streams at Hanford, Washington, USA and Sellafield, Cumbria, UK: The role of iron-sulfide-containing adsorbent materials. *Nucl. Eng. Des.* **226**, 375–385.

Watson, J. H. P., Cressey, B. A., Roberts, A. P., Ellwood, D. C., Charnock, J. M., and Soper, A. K. (2000). Structural and magnetic studies on heavy-metal-adsorbing iron sulphide nanoparticles produced by sulphate-reducing bacteria. *J. Magn. Magn. Mater.* **214**, 13–30.

Weijer, C. J. (2003). Visualizing signals moving in cells. *Science* **300**, 96–100.

Welch, S. A., Taunton, A. E., and Banfield, J. F. (2002). Effect of microorganisms and microbial metabolites on apatite dissolution. *Geomicrobiol. J.* **19**, 343–367.

Wellsbury, P., Mather, I., and Parkes, R. J. (2002). Geomicrobiology of deep, low organic carbon sediments in the Woodlark Basin, Pacific Ocean. *FEMS Microbiol. Ecol.* **42**, 59–70.

Wetzel, D. L., Eilert, A. J., Pietrzak, L. N., Miller, S. S., and Sweat, J. A. (1998). Ultraspatially resolved synchrotron infrared microspectroscopy of plant tissue *in situ*. *Cell. Mol. Biol.* **44**, 145–168.

Wetzel, D. L., Post, G. R., and Lodder, R. A. (2005). Synchrotron infrared micro spectroscopic analysis of collagens I, III, and elastin on the shoulders of human thin-cap fibroatheromas. *Vibr. Spectrosc.* **38**, 53–59.

White, J. L. (1971). Interpretation of infrared spectra of soil minerals. *Soil Science* **112**, 22.

Wildung, R. E., Li, S. W., Murray, C. J., Krupka, K. M., Xie, Y., Hess, N. J., and Roden, E. E. (2004). Technetium reduction in sediments of a shallow aquifer exhibiting dissimilatory iron reduction potential. *FEMS Microbiol. Ecol.* **49**, 151–162.

Yee, N., and Benning, L. G. (2002). *In situ* FTIR study of protonation reactions at the bacteria-water interface. *Geochim. Cosmochim. Acta* **66**, A862.

Yee, N., Phoenix, V. R., Konhauser, K. O., Benning, L. G., and Ferris, F. G. (2003). The effect of cyanobacteria on silica precipitation at neutral pH: Implications for bacterial silicification in geothermal hot springs. *Chem. Geol.* **199**, 83–90.

Yee, N., Benning, L. G., and Konhauser, K. O. (2004a). Silica colloid aggregation by cyanobacteria: A microbial silicification mechanism. *Geochim. Cosmochim. Acta* **68**, A199.

Yee, N., Benning, L. G., Phoenix, V. R., and Ferris, F. G. (2004b). Characterization of metal-cyanobacteria sorption reactions: A combined macroscopic and infrared spectroscopic investigation. *Environ. Sci. Techn.* **38**, 775–782.

Yu, P. (2005a). Molecular chemistry imaging to reveal structural features of various plant feed tissues. *J. Struct. Biol.* **150**, 81–89.

Yu, P., Christensen, D. A., Christensen, C. R., Drew, M. D., Rossnagel, B. G., and McKinnon, J. J. (2004). Use of synchrotron FTIR microspectroscopy to identify chemical differences in barley endosperm tissue in relation to rumen degradation characteristics. *Can. J. Animal Sci.* **84**, 523–527.

Yu, P. Q. (2005b). Application of cluster analysis (CLA) in feed chemical imaging to accurately reveal structural-chemical features of feeds and plants within cellular dimension. *J. Agric. Food Chem.* **53**, 2872–2880.

Yu, P. Q., McKinnon, J. J., Christensen, C. R., Christensen, D. A., Marinkovic, N. S., and Miller, L. M. (2003). Chemical imaging of microstructures of plant tissues within cellular dimension using synchrotron infrared microspectroscopy. *J. Agric. Food Chem.* **51**, 6062–6067.

Zhang, C. L. L., and Lanoil, B. (2004). Geomicrobiology and biogeochemistry of gas hydrates and cold seeps. *Chem. Geol.* **205**, 187–194.

Zhang, Q. H., Piston, D. W., and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895–1897.

Zhang, Y., Yang, M., Dou, X. M., He, H., and Wang, D. S. (2005). Arsenate adsorption on an Fe-Ce bimetal oxide adsorbent: Role of surface properties. *Environ. Sci. Techn.* **39**, 7246–7253.

Zouboulis, A. I., and Katsoyiannis, I. A. (2005). Recent advances in the bioremediation of arsenic-contaminated groundwaters. *Environ. Int.* **31**, 213–219.

This page intentionally left blank

Development and Testing of "On-Farm" Seed Priming

D. Harris

CAZS Natural Resources, University of Wales, Bangor,
Gwynedd LL57 2UW, United Kingdom

   Poor germination and emergence of tropical crops under stressful conditions, such as adverse soil temperature, variable soil moisture, and hardening soils, are major obstacles to obtaining adequate stands of vigorous seedlings and hence reasonable yields in marginal areas of the less-developed world. Representative examples of failures of common tropical crops to establish are reviewed and management techniques appropriate to resource-poor farmers are discussed, including use of good quality seed, dry planting, timely sowing, control of sowing depth, transplanting of seedlings, and seed priming. It is concluded that successful establishment is associated with rapid emergence. Studies of "on-farm" seed priming, whereby farmers can prime their own seeds in water, are reviewed in detail with special reference to work done in developing countries. A large body of *in vitro*, on-station, and participatory on-farm research shows that priming seeds of

129

many important tropical crops in water, typically overnight, before sowing can increase the rate and extent of emergence, improve seedling vigor, advance flowering and maturity, and increase yield in most cases. In some instances, priming does not benefit farmers, but negative effects of priming are rare. The consequences of drying seeds after priming are discussed, as are opportunities for farmers to "add value" to their seeds by using priming to deliver nutrient supplements and increase resistance to pests and diseases. Given its low cost, on-farm seed priming represents good insurance for risk-averse, resource-poor farmers.   © 2006, Elsevier Inc.

I.   INTRODUCTION

More than 2.3 billion tonnes of grains are produced in the world every year (FAOSTAT, 2005). This represents about 60% of all food crops and, excluding clonally propagated crops and fruit and tree crops, it would not be possible without the regular transformation of seeds into plants and back into seeds again. Farmers need an appropriate number of well-spaced, vigorous plants in a field before they can even think of getting a good yield (Brar *et al.*, 1992; Harris, 1996; Radford *et al.*, 1989; Soman *et al.*, 1987) yet good crop stands are often the exception rather than the rule for resource-poor farmers. Observations and surveys, particularly in marginal- and semiarid areas, suggest that poor stand establishment in many crops is common and a major cause of low yields (Soman *et al.*, 1984a). Obtaining an adequate crop stand is the critical management problem that affects much crop production in marginal areas of developing countries (Harris, 1991; Howarth *et al.*, 1997). The higher levels of stand establishment achieved in research trials compared with in farmers' fields are a major factor contributing to the "yield gap" (Shumba *et al.*, 1990).

In marginal rainfed areas, patchy plant stands often result from the failure of the crop to emerge quickly and uniformly. Yields of many crops are reduced because not enough seeds germinate, emergence is slow, and the plants that do eventually emerge are susceptible to drought, pests, and diseases. The constraints facing resource-poor farmers in their efforts to get good stands and reasonable yields under difficult conditions are reviewed in a later section. Some simple, low-input, ways to improve crop establishment are discussed for a range of tropical crops in developing countries. In particular, the consequences of seed priming that farmers can do for themselves "on-farm" are explored in detail.

II.   THE PROBLEM

A.   Inadequate Crop Stands

Harris (1996) reported that, even in researcher-managed trials, around 40% of the sowings in semiarid Botswana failed, often due to poor timing or implementation of sowing operations, but even good quality sowings were prone to failure. In southeastern Zimbabwe, Chivasa et al. (1998) found that 93% of farmers reported poor stand establishment as a problem in maize, while 95% said it was a problem in sorghum and 87% in sunflower while Shumba et al. (1990) noted that the yield gap between recommended practices for maize and groundnut and the situation in farmers' fields was commonly correlated with poor crop stand at harvest.

Many of the crops commonly grown by smallholder farmers in Zimbabwe often need to be replanted two or three times before satisfactory stands are obtained (Chivasa, 1995; Chiduza et al., 1995; Oosterhout, 1996). Chivasa et al. (1998) calculated that enforced serial resowing, coupled with limited local availability of seed, was responsible for much of the within-field sorghum diversity found in the area. Resowing is costly in terms of labor, materials, and reduced yields and often cannot be afforded by smallholder farmers living in marginal areas where poverty and scarcity of resources, such as labor or draft power, are common (Harris, 1991; Willcocks and Twomlow, 1993). Resowing will often lead to a reduction in area planted because seed is often in short supply locally (Chivasa et al., 1998) and consequently to a reduction in household food supply. Strategies that will improve stand establishment with initial plantings will contribute to increased household food security in dry areas (Chiduza et al., 1994). Harris et al. (2001a) reported that the costs associated with resowing are an important reason why farmers in difficult areas of western India get themselves into debt. Droughts are common there, and farmers report crop failure 3 years in 10 and serious shortfalls 4 years out of 10. Farmers identified poor crop establishment as a serious constraint (CDS, 1990).

Small-seeded crops, such as sorghum and pearl millet, predominate in the rainfed arid- and semiarid tropics. Sorghum is grown where mean annual rainfall varies from 600 to 1000 mm, whereas pearl millet replaces it as the dominant cereal crop where rainfall ranges from 200 to 600 mm year^{-1}, both in South Asia (Sivakumar et al., 1984) and in Africa (Spencer and Sivakumar, 1987). Both crops are relatively resistant to the stresses encountered in these harsh environments (Blum and Sullivan, 1986) where the negative effects of low-rainfall totals are compounded by high inter- and intraseasonal variation (Sivakumar et al., 1984). Despite their drought-tolerant characteristics, these crops can still be difficult to establish and

low yields are often due to poor crop stand establishment (Martin and Leonard, 1957). Observations in Siabuwa, Zimbabwe by Chiduza *et al.* (1995) and in Chiredzi, Zimbabwe by Chivasa (1995) confirmed that farmers were regularly using very high seed rates of between 1.2 and 1.8 million seeds per ha, yet plant populations in farmers' fields ranged from 22,000 to 160,000 plants per ha, so fewer than 10% of seeds established successfully. In Niger, West Africa, only 25% of pearl millet seeds sown by hand emerged in farmers' fields and stand failures, attributed to high temperatures, were noted in nearly half of the fields sampled (ICRISAT, 1986). Postemergence seedling survival was also poor, resulting in a further 53% reduction. Establishment of pearl millet in on-station trials was also problematic, with seedling emergence rates ranging from 32% to 40% (ICRISAT, 1987). Yield is reduced, not only because there are too few plants in the field but also because the remaining ones are unevenly spaced, and clumped plants perform less well (Soman *et al.*, 1987). Radford *et al.* (1989) estimated that only 55% of sorghum seed planted in the field in Australia established successfully and that sorghum growers were losing 30% of their potential yield primarily due to inadequate plant densities.

Poor crop establishment is common in other major crops commonly grown in semiarid areas such as cotton (*Gossypium hirsutum*). In a trial series in Sanyati and Sebungwe communal areas in Zimbabwe, data were discarded from 4 of the 11 trial sites because of very poor stand establishment (Cotton Research Institute, 1986). In a separate trial series, data from 50% of sites in Gokwe, Sanyati, and Sebungwe were discarded (Cotton Research Institute, 1996). Mean emergence of common bean (*Phaseolus vulgaris* L.) ranged from 28% to 43% in trials at seven on-station sites across Zimbabwe (Agronomy Institute, 1982), and barley (*Hordeum vulgare* L.) emergence averaged only 26% despite presowing germination scores of 95–98%. According to Chiduza (1987, 1993) and Chivasa (1995), crop establishment failures are common in maize in many areas of Zimbabwe.

B.   FACTORS AFFECTING CROP ESTABLISHMENT

Important physical factors affecting germination and emergence of crops in semiarid areas, alone and in combination, include unpredictable and limited soil moisture, high temperatures, and soil-crust formation. Soil-moisture conditions can fluctuate rapidly as a result of high temperatures and consequent high-evapotranspiration rates (Monteith, 1979; Sivakumar *et al.*, 1984). The temperature a few centimetres below the surface of bare soil is frequently in the range of 40–50°C during the day (Klaij and

Hoogmoed, 1993; Monteith, 1979; Peacock, 1982; Peacock *et al.*, 1990), and the lethal temperature for germination of sorghum, for instance, ranges from 40°C to 48°C (Kailasanathan *et al.*, 1976; Knapp, 1966; Ong and Monteith, 1984; Singh and Dhaliwal, 1972). Exposing sorghum seeds to high temperatures during imbibition slowed germination and reduced the number of seedlings that emerged (Ong and Monteith, 1984), but adverse effects were much less severe when seeds were allowed to imbibe water for 8 h before exposure to high temperatures. Sorghum emergence can be severely affected by high temperatures (Wilson *et al.*, 1982). Even if seedlings emerge, high-soil temperature can result in additional mortality. Peacock *et al.* (1990) described a phenomenon called heat girdling in sorghum whereby the phloem transport system of seedlings is inhibited, thus killing the seedlings.

In maize, Buckle and Grant (1974) showed that temperature of the soil affects emergence in three ways: germination rate and time to emergence are temperature dependent, the potential length of the plumule is restricted by adverse temperature, and morphological abnormalities are induced by wide diurnal temperature fluctuations. The sorghum plumule bends over on reaching a noncrusted surface due to high-surface temperatures (Peacock and Heinrich, 1984).

Soil surface crusts are common in semiarid areas and adversely affect germination and emergence (Grant and Buckle, 1974 for maize; Maiti *et al.*, 1986; Peacock, 1979; Soman *et al.*, 1992 for sorghum). Poor and patchy establishment of cotton is common in Pakistan (Nabi *et al.*, 2001), even where irrigation is available, because many soils form crusts if there is rainfall after sowing but before emergence, and seedlings cannot break through. Seeds sown deep were most affected by crusting because emergence rates were so slow from depth and crust strength increased with time. The emergence of seedlings of pearl millet and chickpea was affected in a similar fashion (Sivaprasad and Sarma, 1987). Emergence through crusts was delayed by 5–6 days as depth of sowing increased from 2 to 8 cm.

Among sorghum genotypes, rapid emergence was correlated with successful emergence through crusted soils (Agrawal *et al.*, 1986). Soman *et al.* (1992) investigated the effect of soil crusts on sorghum emergence and concluded that cultivars with fast germination and high rates of mesocotyl elongation could break through the soil surface before crusts became too strong. Severe soil crusting at Save Valley and Chisumbanje Experiment Stations in Zimbabwe caused poor soybean establishment (Lowveld Research Stations, 1969). Careful, timely, and shallow cultivation to break crusts a few days after planting can improve stand establishment (Grant and Buckle, 1974; Soman *et al.*, 1984) but is not practical for most smallholder farmers because of lack of resources.

III.   SIMPLE WAYS TO IMPROVE
CROP ESTABLISHMENT

Subsistence farmers are well aware of the nature of risk in their production systems. Because they typically have access to only a low level of technology and hence are unable to control environmental factors significantly, they tend to use a variety of tactics to spread risk (Rowland and Whiteman, 1993). Some management strategies appropriate to resource-poor farmers are discussed in a later section and their impact on crop stand establishment highlighted.

A.   SEED QUALITY

High-quality seeds give better stands than poor-quality seeds (Ellis, 1989; Gubels, 1975; Osburn and Schroth, 1989; Parera and Cantliffe, 1994). Gurmu and Naylor (1991) showed that poor-quality seed, imposed by artificially ageing sorghum seeds at high temperature and humidity, germinated poorly and grew slowly, particularly at low-soil water potential.

DeMarco (1990) reported that rates of emergence in wheat were faster from large seeds and from seeds with higher phosphorus content (but not phosphorus concentration), although the effects of seed nitrogen concentration or content were not consistent. Seedling growth parameters, such as leaf size, dry weight, and length of roots, were also increased by increased P-content and larger seeds. Similar results have been reported in other crops (Amjad *et al.*, 2004; Austin, 1972) and DeMarco concludes that increasing P contained in the seed could, at least partially, compensate for the effects of low soil P on early seedling growth. Increasing seed P-content is feasible by fertilizing the seed-producing crop or by soaking seeds in dilute phosphate solutions (see later section). Wheat seedlings from large seeds emerged more rapidly and were taller, heavier, and had more tillers than plants grown from small seeds (Chastain *et al.*, 1995).

Larger seeds of pearl millet were more successful in establishing viable stands of pearl millet in West Africa (Klaij and Hoogmoed, 1993) and in India (Chhina and Phul, 1982) and Onwueme and Sinha (1991) claim that larger seeds are generally more effective, particularly from deeper sowings.

If farmers have access to high-quality seeds (but see Cromwell, 1996) or can select and store their own seed effectively, then they can minimize the negative effects of poor seed quality on crop establishment.

B.   TIMELY SOWING

The probability of good emergence and seedling vigor can be maximized by timely sowing, for example, by planting with the first rains (Fakorede, 1985). Timeliness of land preparation can have important effects on

soil-moisture conservation, mineralization of crop residues, weed growth, and on planting time (Birch, 1960; Rowland and Whiteman, 1993; Stroud, 1985). However, although most farmers are well aware of this, lack of timely access to resources means that, for many, crop establishment remains a compromise between what is desirable and what is achievable (Willcocks and Twomlow, 1993). Poor timing and implementation of sowing operations can result in establishment failures, and the quality of tillage has a big influence (Radford, 1983; Willcocks and Twomlow, 1993). Sowing date can adversely affect stand establishment. For instance, late sowing can expose maturing crops to adverse conditions such as low temperature (Ortiz-Monasterio *et al.*, 1994; Rashid *et al.*, 2004b), high temperature (Musa *et al.*, 2001), and drought (Harris, 2003). Harris (1996) in Botswana reported large differences between sowing dates in emergence and vigor of sorghum seedlings caused by the weather conditions after sowing. If rain fell, or there were humid conditions, immediately after sowing emergence was rapid and seedlings grew vigorously, whereas a period of hot, dry weather delayed emergence and slowed seedling growth and development.

C. Depth of Sowing

There is conflicting evidence on the merits of deep versus shallow sowing (Rao, 1981). For maize, Grant and Buckle (1974) noted that 68% of the seedlings emerged from 5-cm depth, while there was only 20% emergence for seeds planted 10-cm deep. Once the coleoptile sheath in maize had split and the leaves were released underground, the chance of successful emergence depended on the depth of the shoot tip below the surface and the friability of the soil. Deep planting increases time to emergence and consequently may increase exposure to soil-borne diseases (Ellis, 1989; Gubels, 1975; Osburn and Schroth, 1989). Deep planting may also mean that the surface dries and hardens before the seedlings emerge. In contrast, shallow planting places the seed wholly in the zone of greater diurnal fluctuations, both in terms of temperature and soil moisture. Alessi and Power (1971) noted reductions in emergence of maize as sowing depth increased. Generally, the larger the seed, the greater the depth from which it can emerge and thus the deeper it can be sown (Onwueme and Sinha, 1991).

For sorghum, Harris *et al.* (1987) showed that about 90% of seeds sown at 2-cm depth emerged successfully irrespective of soil temperature between 15°C and 25°C, but emergence from 5-cm depth of sowing was reduced to 76% at 25°C and to only 43% at 15°C, that is, only for the deeper sowings was final emergence correlated with temperature and hence rate of emergence. This experiment was conducted in soil in controlled environment glasshouses in which moisture stress was minimized by daily spraying of

the soil surface with water. Harris (1996) compared the emergence of sorghum seeds sown at 3- or 6-cm depth on nine planting opportunities during a single wet season in the field in Botswana, all after substantial rainstorms. Sowing depth had a variable effect on establishment success depending on subsequent conditions. Although seedlings emerged more quickly from shallow sowings when conditions allowed them to do so, that is, when further rain fell or the soil dried out relatively slowly after sowing, they often did not grow as vigorously as those from deeper sowings. Overall, sowing at 3-cm depth resulted in slightly faster and better emergence, whereas sowing at 6-cm depth was associated with a faster rate of leaf production and taller plants at 25 days after sowing (DAS). Given the unpredictability of the postsowing environment, and the ability of sorghum to emerge from up to 10-cm depth (Carter *et al.*, 1992), Harris (1996) concluded that shallow sowings should be avoided if possible. Maiti (1986) measured a wide range of genetic variation in sorghum germplasm for the ability to emerge from depth, and concluded that mesocotyl elongation was related to successful emergence and seedling vigor.

Gill and Prihar (1989) studied the interactions between soil-moisture distribution and sowing depth for barley, chickpea, and wheat and concluded that wheat was the most sensitive to depth of the three crops. Gan *et al.* (1992) studied the effect of sowing depth and seed size on the emergence of wheat in Canada. Results were inconsistent between 2 years but, overall, emergence rate decreased as depth increased, and smaller seeds were slowed more than larger seeds. By tagging individual plants as they emerged, they were able to quantify reductions in yield due to slow rates of emergence. Averaged over 2 years, plants that emerged earliest (1–3 days from "first emergence") produced 1.4 times more grain than plants emerging at 4–6 days and 3.2 times more grain than plants emerging at 7–9 days. The yield differences were largely due to differences in numbers of grain-bearing tillers. Although effects may have been confounded by other issues related to seed size, it is clear that rate of emergence can be important in determining grain yield.

In India, the deep sowing of groundnut seeds reduced shoot biomass, the number and mass of N-fixing nodules, nitrogenase activity, and grain yield (Nambiar and Srinivasa Rao, 1987). They recommended shallow sowings but noted that farmers often sowed seeds deeply to minimize the risk of drought. For pulses, such as chickpea, faba bean, and lentil, Siddique and Loss (1999) showed that, under southwestern Australian conditions, emergence was relatively unaffected by sowing depth and that deep sowings could be beneficial in some circumstances.

Despite conflicting evidence from empirical studies, farmers in marginal areas where soil surfaces are likely to dry rapidly and heat up tend to favor deep sowings to minimize risk. This is particularly the case for larger-seeded crops that are generally better able to emerge from deep in the soil.

D. Dry Planting

Dry planting—sowing seeds in dry soil in advance of expected rainfall—can have benefits that become increasingly obvious as the season progresses (Rowland and Whiteman, 1993) although it depends for its success on a sufficiently definite start of the rains. An early, but light, storm can initiate germination but, if followed by too long a dry period, seedlings may not survive. Dry planting is often used on heavy clay soils, such as Vertisols, that are difficult to work immediately after rain and where long delays can occur before the field is dry enough to plant. In a relatively predictable monsoonal system in India, Lomte *et al.* (1994) showed that, over a 5-year period, dry sowing sorghum outyielded conventional sowing by 24% and also used applied N more efficiently. Although dry planting is attractive in situations where there are shortages, or bottlenecks, of labor or draft power the practice has many drawbacks (Hunt, 1977). Seed can remain in the soil for long periods and may be lost to pests and diseases. If seeds germinate sporadically after light rains, it may not be immediately apparent to farmers that there is a need to resow (Rowland and Whiteman, 1993). Thus, they may delay replanting until they are quite certain that the first attempt has failed. Oosterhout (1996), in her survey in Zimbabwe, found that farmers began replanting in December only when it became clear that the early dry plantings of October and November had failed. Such late planting reduces yields substantially.

Maiti and Moreno (1995) proposed using seed imbibition and redrying as a tool to screen sorghum varieties for the ability to survive partial wetting in the field. They concluded that there was significant genotypic variation, with some varieties able to grow even after 40 h of imbibition followed by 30-h drying and that this ability was associated with the presence of a high concentration of a 33-kDa protein that could form the basis of a screening technique.

Draft power required for cultivation during the dry season is very high for many soils (Willcocks and Twomlow, 1993), and these soils can rarely be tilled to sufficient depth by hand or ox-plough (Rowland and Whiteman, 1993). Ploughing fields after the rains begin is easier and more effective. There may be weed problems in dry planted crops, and successful dry planting is dependent on early weed control.

E. Transplanting Seedlings

Surveys by Chivasa *et al.* (1998) in Musikavanhu communal area in Zimbabwe showed that 97% of the farmers transplant sorghum, using thinnings from overcrowded parts of the field. Factors for successful

transplanting were well known—a moist soil and plants of a particular size. Thinnings were used both to fill gaps and to plant extra areas, particularly if good early rains persisted into the middle of the season. The physiology and agronomic response of sorghum and pearl millet have been investigated in Ghana and Zimbabwe by Young and Mottram (2001) who showed that raising seedlings in small nurseries in advance of the onset of the rains required only small amounts of supplementary water and, when transplanted into the field once the rains had established, those seedlings survived and performed well in comparison to direct-sown crops. Yields of transplanted crops were similar to, or greater than, those of direct-sown crops and flowered and matured much earlier. In farmers' trials in Zimbabwe, all farmers harvested the transplanted crop 3–4 weeks before the direct-sown crop and 89% reported higher yields (Young and Mottram, 2001). Farmers could, if they had access to supplementary water and labor was available, transplant some proportion of their land in this fashion as a way of extending the growing season in short-duration rainfall areas, thus spreading risk to improve food security.

F. Seed Priming

Soaking seeds in water before planting is not new. Evenari (1980) reviewed the history of germination research and noted that, for example, farmers in ancient Greece soaked cucumber seeds in water or milk with honey before sowing to increase germination rate and emergence. Wilkinson (1918) recommended the placement of seeds of radish, bean, corn, and cucumber in lukewarm water overnight to increase germination velocity. Over the last 40 years, priming seeds with various substances has become a common seed treatment to increase the rate and uniformity of emergence in many vegetables, flowers and, sometimes, field crops. There is now a large literature on the effects of seed pretreatments on seed quality, germination, crop establishment, growth, and yield. These treatments have been characterized and reviewed many times (Ashraf and Foolad, 2006; Hegarty, 1978; Heydecker and Coolbear, 1977; Khan, 1992; Parera and Cantliffe, 1994; Taylor et al., 1998).

Almost all the technologies considered by these reviewers have been developed with the assumption that seeds with "added value" can readily be accessed by farmers through formal seed supply chains. Adding value to seeds is often quite complicated or requires special equipment. Such methods, mostly developed for temperate situations, rely on carefully controlled hydration usually involving aeration to avoid imbibition damage (Ellis et al., 1990; Legesse and Powell, 1992; Matthews, 1980) and include use of solid matrix materials (Taylor et al., 1988), imbibition controlled

using sophisticated monitoring, such as drum priming (Rowse, 1996) a variety of osmotically active compounds (Brocklehurst *et al.*, 1984; Khan *et al.*, 1978) and inorganic salts (Paul and Chaudhury, 1991) generally followed by rapid redrying of seeds before storage and or distribution to farmers.

Cromwell (1997) estimates that only around 10% of the seed needs of farmers in developing countries are met by formal (public and private commercial) seed systems. Similarly, according to Jaffee (1991), over 80% of the seed of staple crops in developing countries is farm-saved. In India, Pal *et al.* (2000) reported that, in rice, official estimates of annual seed turnover rate (i.e., the proportion of seed that is replaced every year from the formal sector), based on average figures between 1995 and 1998 ranged from almost zero in Tamilnadu State to 32% in Andhra Pradesh State. The national average was only 14%, a figure confirmed by Turner (1994), and this in a crop grown on almost 50 million ha (Subbarao *et al.*, 2001). The national average for wheat was even lower, at 7% (Turner, 1994). A similar situation occurs in Africa where maize seed in a few countries was relatively regularly available to farmers through the formal sector, whereas in most countries the proportion of nonformal-source seed was high (Table I).

Cromwell (1996) asserts that the availability of seed per se can affect African farmers' ability to sow a crop at all, and Chivasa *et al.* (1998) demonstrated that farmers in Zimbabwe were often constrained by poor seed availability in the choice of which varieties of sorghum to grow. Access to the formal sector affords an opportunity to market "added value" seeds (although it must be noted that "added value" invariably also means "added cost"), but the reality is that most seed is farm-saved or from local, informal sources. Clearly, if farmers are to gain benefits from "enhanced" seed then they need appropriate methods, that is, those that they can apply themselves to their own seed. Resource-poor farmers cannot control imbibition except by timing, find aeration of solutions difficult and cannot use sophisticated drying techniques. Consequently, unless noted otherwise, this chapter only considers work on priming by soaking in nonaerated free water, followed either by immediate sowing or by air drying and henceforth termed "on-farm" seed priming (Harris, 1996), and it also emphasizes work done in developing countries.

IV. "ON-FARM" SEED PRIMING

Once sown, seeds spend significant amounts of time absorbing water from the soil. By reducing this time to a minimum, seeds can be made to germinate, and seedlings made to emerge, more quickly. Farmers can prime their

Table I
Estimates of Maize Seed Markets in Some African Countries

Country	Area (1000 ha)	Quantity of seed planted (t)	Seed purchasing frequency (%)
Nigeria	4702	117,550	20
South Africa	3770	60,320	75
Zimbabwe	2000	50,000	75
Ethiopia	1800	45,000	20
Tanzania	1602	40,050	20
Kenya	1360	34,000	75
D.R.C.	1349	33,725	10
Malawi	1235	30,875	33
Mozambique	1081	27,025	10
Egypt	774	19,350	85
Cote d'Ivoire	692	17,300	10
Ghana	667	16,675	25
Zambia	599	14,975	50
Angola	596	14,900	15
Uganda	585	14,625	20
Benin	479	11,975	10
Togo	354	8850	10
Somalia	350	8750	10
Morocco	327	8175	20
Cameroon	317	7925	25
Mali	237	5925	10
Madagascar	189	4725	10
Burkina Faso	180	4500	10
Burundi	113	2825	10
Lesotho	106	2650	33
Swaziland	60	1500	80
Rwanda	51	1275	10
Total		605,445	

Data from MacRobert, J., CIMMYT, Harare (personal communication).

own seed if they know the "safe limits"—the maximum length of time for which seeds can be soaked and which, if exceeded, could lead to seed or seedling damage. These safe limits can be calculated for each variety so that germination will not take place before sowing after seeds are removed from the water. Primed seed will only germinate if it takes up additional moisture from the soil after sowing. It is important to note this distinction between priming and pregermination—sowing pregerminated seed under dryland conditions can result in total failure to emerge.

For many tropical crops, "overnight" is well within the safe limits for priming. Farmers can prime seed overnight and simply surface dry it before sowing. Apart from swelling slightly and weighing more, primed seed can be treated in the same way as nonprimed seed. If sowing is delayed, for example,

by heavy rain, it can be surface-dried and stored in a dry place for several days without loss of viability. For the purposes of this review, the definition of "on-farm" seed priming is extended to include seeds air dried after priming because, in practice, sowing is frequently delayed (see in a later section).

A.   In Vitro Investigations of Rate and Extent of Germination

Harris and Mottram (2005) tested the germination response to seed priming of the following crops: wheat, chickpea, cowpea, maize, upland rice, mungbean, mustard, sorghum, pearl millet, and finger millet. Seeds from a number of varieties (from 2 to 17) of each crop were tested in an incubator at constant temperature. Crops normally sown under summer conditions were tested at 30°C (and also at 40°C for pearl millet) whereas winter-sown crops were tested at 20°C. After preliminary experiments to determine optimum priming durations for each crop, 50 seeds of each variety were either immersed in distilled water or kept dry at the same temperature. Soaked seeds were removed from the water, surface dried and set to germinate at the same temperature on moist filter paper in petri dishes or lidded plastic containers, depending on seed size. The authors concluded that priming for 8–12 h ("overnight") resulted in significant reductions in the time for 50% of the seeds to germinate ($t_{g50\%}$) in most varieties of all crops, with no significant differences in final emergence percent between primed- and nonprimed seed lots of any given crop or variety. Eight hours was suggested as the most appropriate soaking duration for all crops tested as it equates to "overnight" for farmers, although they acknowledged that wheat, maize, and rice can be primed for up to 18 h without problem.

1.   Wheat

Although their study was primarily an investigation of the effect of priming wheat seeds with various concentrations of gibberellic acid, the results of Parashar and Varma (1988) clearly demonstrate that soaking in water for 6, 12, and 18 h, followed by drying for 3 h, increased rates of germination in both nonsaline and saline conditions.

Harris et al. (2001b) compared the germination characteristics at 20°C, and the response to priming with water, of 12 wheat varieties from South Asia. Final germination percentage was not affected by priming seeds for 8 h or by a treatment in which seeds were primed for 8 h then dried for 24 h. However, the mean $t_{g50\%}$ was reduced from 51 to only 27 h (47%) following priming for 8 h. Seeds dried for a further 24 h after priming retained the

ability to germinate faster than nonprimed seeds, although the advantage was reduced to a $t_{g50\%}$ of 43 h.

Rashid *et al.* (2002) reported an experiment in which the germination (in Petri dishes with 200 mol m^{-3} NaCl to simulate saline conditions) of wheat variety KRL 1-4 was measured in response to priming with water for 8 h. Neither priming nor salinity had any significant effect on final germination percentage, but $t_{g50\%}$ was affected by both factors. The $t_{g50\%}$ was 52 h for nonprimed seeds in nonsaline conditions but only 35 h for primed seeds. Germination of nonprimed seeds in 200 mol m^{-3} NaCl was very slow (99 h) and priming reduced this to 62 h.

2. Upland (Direct-Seeded) Rice

Harris and Jones (1997) tested the germination response to seed priming of 11 varieties of upland rice, including traditional and improved *O. sativa* and *O. glaberrima* varieties and new interspecific hybrids (Jones *et al.*, 1997; WARDA, 2002). Priming seeds with water for 24 h did not affect the final germination percentage, but it reduced $t_{g50\%}$ in all varieties from a mean of 46 h down to 32 h. The actual time saved by priming varied from 7 to 20 h. Harris and Mottram (2005) tested 10 varieties primed for 12 h and reported significantly faster germination. However, there was no significant relation between the faster germination caused by priming and the nonprimed germination rate for priming durations of either 12 or 24 h (Harris and Mottram, 2005).

Harris *et al.* (1999) used the popular Indian upland rice variety Kalinga III to test the effect of various combinations of priming duration and post-priming dry storage on $t_{g50\%}$ at 30°C. Again, none of the priming treatments affected final germination percentage but priming for 12, 24, and 36 h in water reduced $t_{g50\%}$, although the reduction by priming for 36 h was little better than that of 24 h. Keeping seeds dry for 24 h reduced the benefits from priming but $t_{g50\%}$ in all cases was still less than that for nonprimed seeds.

3. Maize

Germination of two Indian varieties of maize (Sameri and Shweta) was significantly hastened at 30°C ($t_{g50\%}$ reduced from 38 to 19 h) following priming with water for 24 h. However, the primed seeds continued to germinate even after they were kept dry for a further 24 h, suggesting that the safe limit for priming had been exceeded. Soaking seeds for 8 h at 20°C reduced $t_{g50\%}$ from 86 to 50 h (Harris *et al.*, 1999). Harris *et al.* (2002a) showed that priming for 12 h significantly reduced $t_{g50\%}$ in 17 out of 18 maize cultivars from Zimbabwe. Final germination was not affected by priming.

Further analysis by Harris and Mottram (2005) concluded that the proportional response to priming was greater in cultivars that were inherently slower to germinate without priming. Rashid *et al.* (2002) noted faster germination under both saline (100 mol m^{-3} NaCl) and nonsaline conditions following priming for 24 h in maize cv. Sarhad White, but only under nonsaline conditions in cv. Dehquan. Germination studies involving a range of substrate water potentials (Murungu *et al.*, 2005) confirmed that final germination percent of maize decreased as water potential decreased from 0 to –1500 kPa and that seed primed for 12 h was less sensitive to moisture stress than nonprimed seed, that is, the relative effect of priming increased as moisture stress increased.

4. Sorghum

Harris (1991, 1996) reported data from germination experiments with sorghum cv. Segaolane in controlled environments and showed that $t_{g50\%}$ at 30°C decreased as the soaking time increased from 0 to 10–12 h, a treatment in which a 50% saving in time could be achieved. However, germination of seeds soaked for longer than 10–12 h was found to continue even after soaking ceased, and so would be susceptible to damage during sowing operations or in the event of any delays in sowing. Al-Soqueer (2004) tested the germination of two sorghum varieties from Saudi Arabia and found that soaking seeds in water for 12 h increased both the $t_{g50\%}$ and the final germination if sown immediately, whereas drying for 24, 48, and 72 h had no effect on final germination but increased $t_{g50\%}$ slightly relative to the nonprimed control.

5. Cotton

Murungu *et al.* (2005) concluded that germination of two cotton varieties (CY889 and SZ93–14) was inhibited progressively as water potential of the medium (aqueous polyethylene glycol solutions) was reduced, that is, made more negative. There was no significant effect of priming for 12 h on germination rate or final germination at water potentials of 0 or –10 kPa, but priming increased both significantly ($p < 0.05$) at higher stress levels of –50, –100, and –500 kPa.

6. Chickpea

The germination characteristics and response to priming at 20°C of the Indian chickpea cultivars ICCV 2, ICCV 10, ICCV 88202, GL 769, and L 551 were studied by Harris *et al.* (1999). The mean $t_{g50\%}$ was reduced from

around 50–35 h by priming for 8 h, but there was some evidence that primed seeds, particularly, those of ICCV 2, ICCV 10, and L 551, tended to continue to germinate after priming had finished. In a small plot experiment, emergence of cv. ICCV 10 and cv. Dahod Yellow was significantly reduced from about 230 to 190 h by priming seeds for 8 h.

7. Bambara Groundnut

Linnemann and Azam-Ali (1993) noted that yield of bambara groundnut was often constrained by poor germination, emergence, and stand establishment. Massawe *et al.* (1999) recorded the imbibition time course for three bambara groundnut landraces and, although there were differences in the rates at which varieties took up water, final germination percentage was higher for primed seeds than for nonprimed seeds. The optimum soaking time was 24 h for two of the varieties tested but 72 h for the third one. In contrast, Mabika (1992) found no effect of priming seeds for 24 h on the germination behaviour of two other landraces.

B. *In Vitro* Emergence and Early Seedling Growth

1. Wheat

Bhati and Rathore (1986) soaked wheat (cv. Sonalika) seeds in India in distilled water for 14 h and then dried them at room temperature for 4 h. Priming resulted in significantly ($p < 0.05$) faster emergence and 11% more seedlings that were 22% taller with shoots that were 18% heavier on a dry weight basis 20 DAS. Seedlings from primed seeds had 18% longer roots ($p < 0.1$).

Final emergence of seedlings of wheat variety KRL 1–4 from soil in pots was unaffected by either priming with water for 8 h or by saline conditions, but the time for 50% emergence ($t_{e50\%}$) was reduced from 108 to 95 h by priming under nonsaline conditions and from 166 to 147 h when irrigated with 150 mol m^{-3} NaCl (Rashid *et al.*, 2002). These results confirmed those of Idris and Aslam (1975) who soaked wheat seeds for 12 and 24 h, air dried them (time not specified) to "about" their original weight, and found that germination rate in water and in 0.5% NaCl was enhanced by priming with water and primed seedlings were taller with heavier roots and shoots.

2. Upland (Direct-Seeded) Rice

Fast germination, emergence, and rapid development of ground cover are important traits for upland rice varieties grown in West Africa where

competition from weeds is a major constraint on yield (Adesina *et al.*, 1994). Harris *et al.* (2002b) described two *in vitro* experiments with rice to investigate the consequences of early emergence for seedling vigor and, by implication, competition with weeds. They simulated the effects of rapid emergence of rice plants on competition with other, adjacent, rice plants behaving as weeds and emerging at the same time as, or later than, the crop plants. Although the total fresh biomass (sum of 10 crop plants plus 10 "weed" plants) of 25-day-old seedlings in the containers remained the same irrespective of when the weed plants had emerged (i.e., 0, 1, 2, or 3 days after the crop plants), and this biomass was significantly greater than that produced by 10 crop plants without weed competition, crop biomass increased as the delay in weed emergence increased. In contrast, the weed biomass declined with each successive delay. Thus, plants that emerged earliest were able to sequester more resources and grew faster at the expense of plants emerging later. This effect probably forms the basis for the farmers' observations concerning better weed suppression by primed plants reported by Harris *et al.* (1999).

3. Maize

Results of a similar experiment to that described by Harris *et al.* (2002b) for rice but with maize are shown in Fig. 1 and Table II. As for rice, it was assumed that the most effective competition for maize plants would be other maize plants since they occupy exactly the same ecological niche. Consequently, seeds of cv. R201 maize were sown in soil at field capacity in large trays (0.5 m × 0.35 m × 0.18 m) in a greenhouse as shown in Fig. 1. Ten seeds per row were sown and seedlings thinned on emergence to leave five regularly spaced plants at positions A and B. "A" plants were designated the "crop" and sown on day 0, while "B" plants were designated as "weeds" and were sown on day 0, 1, 2, or 3 according to treatment. This was to simulate delayed emergence of nonprimed "weed" seed relative to primed "crop" seed since farmers had noted that primed crops exhibit great early vigour and, in some circumstances, compete aggressively with weeds (Harris *et al.*, 1999). They commonly report faster emergence of 1–3 days following seed priming, hence the choice of treatments used. A further treatment contained only four "A" rows and represented the "no competition from weeds" situation, in which only within-crop competition would be expressed.

The effect of treatment on the "crop" ("A" rows) was highly statistically significant for all four traits listed in Table II. In relation to "crop" and "weed" emerging at the same time (treatment 0, i.e., maximum competitive pressure), 1 day's delay (treatment 1) in emergence of the "B" rows did not significantly increase the development or growth of the "A" rows. However,
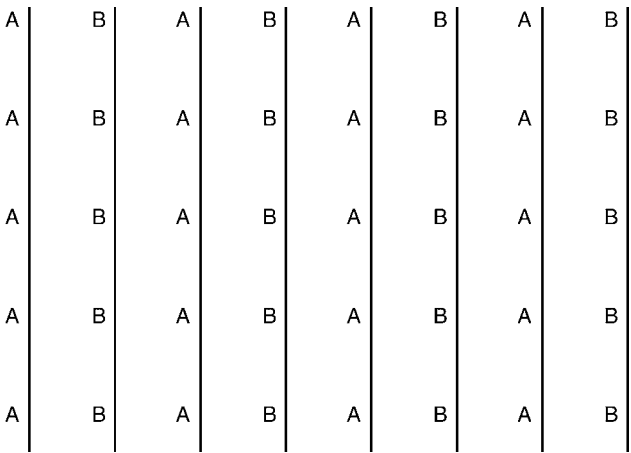
Figure 1 Planting design for competition experiments using R201 maize. A = "Crop"; B = "Weed." B rows were sown 0, 1, 2, or 3 days after the A rows.

a 2- or 3-day delay in the emergence of the "B" rows significantly increased the number of leaves per plant, plant height, number of root axes per plant, and fresh weight of the "A" rows relative to a 0- or 1-day's delay. "A" row plants growing in the absence of "B" rows (no competition) were significantly taller and heavier than in all other treatments. Conversely, the performance of the "B" rows was best when they emerged at the same time as the "A" rows and declined in proportion to any delay. Again, there was no significant difference between 0 and 1 day's delay, except in leaf number per plant (Table II).

The data from this artificial system of competition suggest that early comparative emergence of maize (and also upland rice as described by Harris *et al.*, 2002c) and the resultant rapid early growth and development might confer some competitive advantage over weeds that emerge at the "normal" time, irrespective of any physiological advantages that priming might confer. The critical delay appears to be between 1 and 2 days. If primed maize seeds do emerge significantly earlier than otherwise, a mechanism seems to exist for a competitive advantage over weeds that might explain some of the benefits in the field reported by, for example, Harris *et al.* (1999, 2001a) and Chivasa *et al.* (1998).

The upper limit for priming in maize was determined as 24 h in India in a field emergence experiment reported by Harris *et al.* (1999) and in a pot experiment in Zimbabwe by Chivasa *et al.* (2000). These latter authors also noted significantly faster emergence, taller and heavier seedlings, and more leaves per plant (all measured 14 DAS) from seeds primed for longer than

Table II
Mean Values and Significant Differences for Four Traits

Trait	Delay (days)	Mean value	
		"Crop" (A)	"Weed" (B)
Leaf no. per plant	0	3.6^a	3.6^a
	1	3.7^a	3.3^b
	2	3.8^b	3.1^c
	3	3.9^b	3.0^c
	No comp.	3.9^b	
	LSD(0.05)	0.14	0.19
Height (cm)	0	51.8^a	51.7^a
	1	53.8^a	49.6^a
	2	56.4^b	43.9^b
	3	57.7^b	40.8^b
	No comp.	61.5^c	
	LSD(0.05)	2.14	3.61
No. of root axes per plant	0	9.3^a	9.5^a
	1	9.3^a	9.2^a
	2	10.0^b	8.4^b
	3	9.9^b	7.9^b
	No comp.	10.5^b	
	LSD(0.05)	0.57	0.65
Fresh weight per 20 plants (g)	0	81.2^a	72.4^a
	1	87.0^a	63.6^a
	2	102.6^b	48.3^b
	3	114.2^c	41.6^b
	No comp.	136.1^d	
	LSD (0.05)	8.50	8.98

All measured 25 days after sowing (from day 0) in the "A" Rows and the 'B' Rows separately. "No comp." = "A" rows grown without "B" rows. Within each trait and column, values followed by the same letter do not differ significantly at $p < 0.05$. Values are taken from 4 rows × 5 plants = 20 plants.

8 h in cv. R201. In contrast, these variables were not significantly increased in cv. PAN 6363 unless seeds had been primed for at least 16 or 18 h. The number of root axes per plant was not increased, relative to the nonprimed treatment, until seeds had been primed for at least 14 h in cv. R201 and for 20 h or more in cv. PAN 6363. Harris (1996) has noted a correlation between rate of formation of root axes and seedling vigour in sorghum.

Finch-Savage et al. (2004) investigated the interactions between temperature during priming and during germination and emergence of maize from sand cores differing in moisture content. In dryer conditions at 35/28 °C day/ night temperatures, priming for 17 h reduced emergence time and increased final emergence percentage relative to nonprimed seed. In moist cores,

priming advanced emergence at 30/20°C but reduced final emergence at 35/28°C and both delayed and reduced emergence at 40/28°C. Priming also increased the sensitivity of seeds to high temperatures, decreasing the optimum and ceiling temperatures for germination. The authors noted that responses sometimes differed between seedlots and aerating the soak water or allowing primed seeds to dry for 1–2 h mitigated the negative effects of high temperatures and wet conditions.

Murungu *et al.* (2003), in pot studies with drying soils in Zimbabwe, primed seeds of maize cv. SC401 and consistently improved emergence and early seedling growth across a range of soil aggregate sizes (<1, 1–2, and 2–4.75 mm, 4.75–16 mm) and initial soil matric potentials (–10, –50, and –100 kPa). The number of seedling root axes measured 8 DAS was increased by priming at all matric potentials and in all aggregate sizes >1 mm. In finer soil (<1 mm) root number was decreased by priming in the wetter soils (–10 and –50 kPa) but not at –100 kPa. Priming also increased shoot length 8 DAS significantly from 48 to 96 mm at –10 kPa, 19–31 mm at –50 kPa and from 3 to 13 mm at –100 kPa. The authors concluded that seed priming in maize can partly compensate for the negative effects of low-soil water matric potential on crop establishment, but they also introduced a note of caution when primed seed was sown in wet conditions where aeration was likely to be restricted.

4.   Sorghum

Emergence of sorghum cv. Segaolane at 30°C from soil in trays was significantly hastened by 23% when seeds were presoaked for 6 h or longer (Harris, 1991, 1996). The rate of imbibition during soaking was found to be proportional to temperature, and was faster in free water at 20°C, 30°C, or 40°C than in moist soil at 30°C, but soaking for 8–10 h was recommended for sorghum crops sown in southern Africa.

Chivasa *et al.* (2000) measured the emergence and seedling growth of two varieties (Red Swazi and Muchayeni) of sorghum in a pot experiment in Zimbabwe and concluded that soaking sorghum for 12 h or more caused more than 50% of seeds to sprout before sowing. For soaking durations of 8 and 10 h, priming hastened the onset of emergence in both cultivars relative to nonprimed seeds but 10 h was generally better than 8 h. The $t_{e50\%}$ was reduced significantly ($p < 0.001$) by 23% and final emergence percent 14 DAS was significantly ($p < 0.05$) higher after 10 h priming, relative to not priming, in both cultivars. Fourteen-day-old seedlings, of both cultivars, from seeds primed for 10 h also had significantly ($p < 0.001$) more leaves and root axes and were taller and heavier than nonprimed seedlings.

Emergence from soil in pots at 30°C after soaking in water for 12 h was 26% better than not priming if primed seeds were sown immediately. Drying

for 24, 48, or 72 h reduced this advantage to 10–13% (Al-Soqueer, 2004). Emergence rate was increased by priming without drying by 16%, but no benefit remained after primed seeds were dried. Root dry weight measured 15 DAS was significantly increased by priming (without drying) but shoot dry weight was not. Seedlings from seeds primed then dried were not significantly different from nonprimed plants. When tested across a range of salinity, emergence rate from pots was increased by priming (without drying) by about 60% at 0.4 and 4 mmhos cm^{-1} and about 35–40% at 8, 12, and 15 mmhos cm^{-1}, although differences at 12 and 15 mmhos cm^{-1} were not statistically significant. Priming increased root dry weight measured 25 DAS across all salinity levels, by a mean of 25% but shoot dry weight was not affected, relative to not priming. Emergence rates were also enhanced by priming by about 50% across a range of soil bulk densities from 1 to 1.6 g cm^{-3}.

Priming for 12 h increased emergence rate (by 18%) and final emergence (by 25%) from soil in a glasshouse at 30°C but only if sown immediately after priming. Seeds dried for 48 h after priming did not differ from nonprimed seeds (Al-Soqueer, 2004). Plant height and shoot dry weight were increased by priming (without drying) and grain yield was increased significantly ($p < 0.05$) by 16% as a result of increases in the number of grains per panicle and grain size. Similar results were obtained in a second year with a 34% yield increase due to priming.

5. Cotton

Murungu *et al.* (2003) showed that, in pot studies using a range of soil matric potentials at sowing and a range of soil aggregate sizes, emergence rate and final emergence percent of cotton seedlings decreased as water potential decreased and there was no emergence at –200 or –1500 kPa. Although there was no significant effect of priming on emergence rate, it significantly increased final emergence from 75% to 99% at –10 kPa, from 7% to 79% at –50 kPa, and from 1% to 7% at –100 kPa. Primed cotton seedlings, measured 8 DAS, had longer roots and shoots than nonprimed seedlings at all initial matric potentials. The authors concluded that priming could partly compensate for the negative effects of drying soils on emergence and early growth.

6. Bambara Groundnut

In emergence studies (Massawe *et al.*, 1999), priming produced heavier seedlings and larger leaf areas measured at 14 DAS and also at 20 DAS.

Measurements between 8 DAS and 20 DAS suggested that priming-related differences were not just a function of earlier emergence (and hence growth for a longer period) but were also associated with faster relative growth rates.

C. RESEARCH STATION STUDIES

1. Wheat

There are few examples of investigations of priming in wheat that do not involve drying primed seeds back to storage-moisture content. In an early paper from India, Dhingra *et al.* (1974) soaked wheat seeds for 18 h (without drying) and improved grain yield by about 200 kg ha^{-1} and straw yield by about 400 kg ha^{-1}. Dayanand *et al.* (1977) included a comparison of the effects of priming overnight, without drying, on the uptake of N, P, and K by seven varieties of wheat in the field. Priming had no significant effect on the uptake of P and K but significantly increased the uptake of N by 8% and 7% in the two consecutive years of the study. The authors attribute this effect to faster germination and growth in plots with primed seed, but no yield data were given so it is not possible to say if the extra N resulted in the production of more grain or straw. Bhati and Rathore (1986) soaked wheat (cv. Sonalika) seeds in India in distilled water for 14 h and then dried them at room temperature for 4 h. This drying period was probably not long enough for seeds to regain their original moisture content and is of a similar order to delays after priming that might be encountered in farmers' fields. Priming resulted in significantly ($p < 0.05$) faster emergence and bigger seedlings measured 20 DAS. Although priming had no effect on the tissue content of N, P, and K, it did increase the uptake per seedling, presumably as a consequence of their larger size, of N by 18% ($p < 0.05$) and P (20%) and K (18%) (both $p < 0.1$) relative to nonprimed plants. As in Dayanand *et al.* (1977) no yield data were given. Mandal and Basu (1987) demonstrated that priming in water for as little as 2 h (followed by redrying to storage-moisture content) both as a mid-storage treatment and as a presowing intervention could increase grain yield significantly.

Sen and Misra (1984) and Paul and Choudhury (1991) present yield data but there is some uncertainty concerning whether seeds were dried after priming and, if so, for how long. Sen and Misra (1984) do not give any details of how priming was achieved but reported a significant ($p < 0.05$) increase in grain yield of 14% and 13% relative to crops from nonprimed seed in two consecutive years in India. Paul and Choudhury (1991) in Assam primed wheat seeds for 18 h and then dried it ''in the shade'' (duration unspecified) ''to bring back to *almost* (my italics) original weight.'' Averaged over two varieties (WH 291 and Sonalika) priming increased grain yield

significantly ($p < 0.05$) by 15% and 25% in two consecutive years and straw yield by 8% and 16%.

Rashid *et al.* (2002) primed wheat seeds overnight in two RBD trials at Gundheri in Pakistan and significantly increased grain yield by 22.5% ($p < 0.05$) and 24.3% ($p < 0.10$).

2. Upland (Direct-Seeded) Rice

Singh and Chatterjee (1981) included soaking seeds of three varieties of upland rice in water for 24 h, followed by air drying, in their trial in India of 11 seed treatments. Priming seeds for 24 h significantly improved stand establishment, relative to nonprimed seeds, by 43%, 24%, and 23% in three consecutive years and increased grain yield by 11%, 24%, and 20%, respectively.

Harris *et al.* (2002b) compared the effects of seed priming on the yield of 10 upland rice varieties in 2 years of on-station trials in Rajasthan, India. Averaged over all varieties, priming seeds overnight in water increased grain yield by 18% in 1997 and by 40% in 1999, a much drier year. Components of yield were measured in 1997 and results showed that the increased yield was due to a combination of faster emergence and better crop stands (91% emergence versus 61% in nonprimed crops) and better individual plant performance. Mean time to flowering was reduced by priming by almost 4 days, plant height was increased from 94 to 108 cm and the number of panicles per plant was increased from 4.9 to 5.7. An additional treatment, that is, priming seeds with a 2% solution of NaCl, improved yield by less than priming with water alone.

Priming rice seeds for 12 and 24 h was tested in Ghana in 2000 on the research station of the Crops Research Institute, Fumesua (WARDA, 2002, pp. 21–29). Priming resulted in significantly faster, more complete crop establishment, more vigorous growth (larger leaf area, taller plants, and higher root and shoot dry weights measured 4 weeks after sowing) in both varieties that were tested. Primed plants also produced significantly more tillers, panicles and grains per panicle than nonprimed plants. There was little difference between priming for 12 or 24 h and the mean yield increase over the nonprimed treatment was about 25%.

3. Maize

Work in Zimbabwe suggests inconsistent responses of maize to priming (Murungu *et al.*, 2004a) in a study where the crop was established by over-sowing and thinning back to a target population. Priming increased the rate of emergence relative to nonprimed seed in 8 out of 9 year $\times$ simulated

rainfall combinations. In the ninth comparison, associated with very high soil temperature, priming slowed emergence. There was no significant effect of priming on final stand nor on relative growth rate and, although priming significantly reduced time to maturity from 113 to 100 days in 1 of the 2 years, there were few meaningful effects on other components of yield and no yield increases in either year. Since Murungu *et al.* (2004b) concluded from a related study at the same site that yield benefits from priming in maize were mainly a consequence of better crop stands, the negative results of Murungu *et al.* (2004a) are not unexpected.

Harris *et al.* (2004) summarized the results of 14 trials of maize-seed priming in Pakistan over 4 years between 1998 and 2000. Priming for 16–18 h was found to be the optimum duration and gave statistically significant ($p < 0.05$) increases in grain yield, ranging from 17% to 76%, in 11 out of the 14 trials. In no case was the result of priming worse than not priming.

4. Sorghum

In two field sowings in Botswana in 1991–1992, priming sorghum seed for 10 h in water gave large increases in emergence, seedling development, and growth over the first 25 DAS (Table III). Plots with primed seed had significantly more

Table III
Effect of Seed Treatment on Sorghum Variables Measured 25 DAS in a Field Emergence Experiment in Botswana, 1991–1992

	Seed treatment	
Variable	Not primed	Primed with water for 10 h
Emergence		
Emergence (%)	13.4	24.7(84)
Development		
No. of leaves (pl^{-1})	5.5	6.2(11)
No. of root axes (pl^{-1})	6.6	8.1(22)
Growth plant^{-1}		
Plant height (mm)	58	72(23)
Shoot dry matter (mg pl^{-1})	452	712(58)
Growth per unit area		
Shoot dry matter (kg ha^{-1})	10.9	34(214)
[Seed rate ($\times 10^3$ ha^{-1})]	139	181(30)
Shoot dry matter adjusted for sowing rate (kg ha^{-1})	10.9	27.8(154)

Data are from Harris, D., and Patrick, C. (unpublished). Values are means of two sowing dates. The relative advantage of the priming treatment over nonprimed seed is given as a percentage in parentheses.

plants and those plants produced leaves and roots faster and were taller and heavier. After accounting for systematic differences in sowing rate between treatments (because primed seed swelled and ran through the planter at different rates), shoot dry matter per unit area was increased by 154% in the primed treatment. Final harvest comparisons were planned, but the 1991–1992 season was the driest since records were kept in Botswana. The primed plots produced some grain whereas the control plots all failed, but all the grain was consumed by birds that had no other source of food in such a dry year.

Al-Soqueer (2004) investigated the interaction between priming and three soil moisture regimes, using differential irrigation, in a field experiment in Saudi Arabia. Overall, emergence was only around 50–60% but priming for 12 h (without subsequent drying) increased emergence by 14% and was associated with a 10% increase in emergence rate. Neither the emergence percent nor the rate of emergence of nonprimed seeds and seeds dried for 48 h differed significantly. Root dry weight measured 30 DAS was 50% larger in the primed (without drying) treatment, averaged across all moisture regimes. Shoot dry weight was increased by priming (without drying) at 20, 40, 60, 80, and 95 DAS and under all moisture regimes except the driest at 80 and 95 DAS. Priming and drying seeds for 48 h gave no benefit relative to not priming. Grain yield was increased by 18%, associated with significant increases in panicle number per unit area, grain number per panicle, and 1000-grain weight.

5. Pearl Millet

The practical implications of sowing seeds into soils with low moisture are demonstrated by the data shown in Fig. 2. This field experiment was conducted on a sandy soil in Rajasthan, India in the dry off-season and emergence, even in moist soils [volume moisture content (VMC) = 9.4%], was only around 50% despite all seed lots used having germinability over 90%, thus confirming the large differences between field emergence and laboratory-based germination testing reported in marginal environments. For instance, Chiduza et al. (1995) and Chivasa (1995) reported that less than 10% of sorghum and pearl millet seeds established successfully in farmers' fields in Zimbabwe. In Fig. 2, as soil moisture content at sowing declined (with increasing distance from a presowing irrigation line source), the emergence percentage of nonprimed seeds also declined until only about 12% of seeds sown at a VMC of 6.7% emerged. Primed seeds, however, emerged better at all levels of soil moisture and the relative increase due to priming increased from 15% in moist soil to 45% in dry soil. Priming was not able to compensate completely for the effects of low-soil moisture at sowing but made a significant contribution across a range of soil moisture contents and was relatively more effective in drier soils.
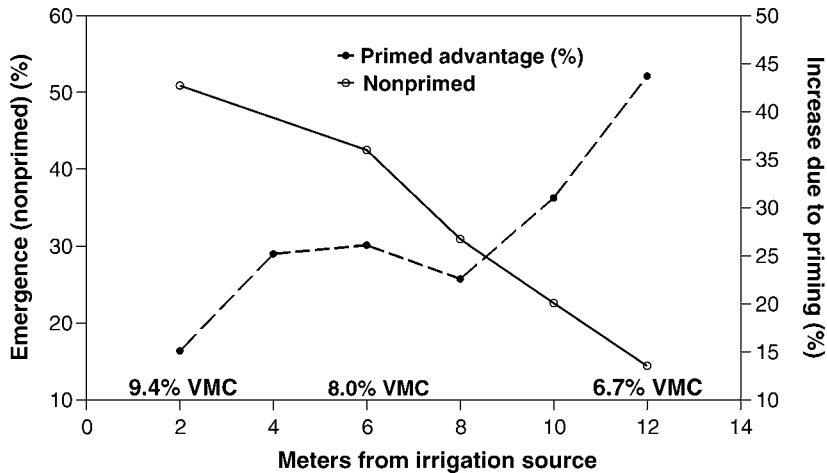
Figure 2 Mean emergence percentage (open symbols, left-hand axis) of pearl millet seedlings from nonprimed seeds and the percent increase (filled symbols, right-hand axis) due to priming seeds in water for 8 h, in relation to increasing distance from an irrigation sprinkler line used to wet soil prior to sowing. Volume moisture content (% VMC) at sowing is shown for 3 distances from the line source. Data are means of eight open-pollinated varieties sown in the field in Fatehpur, Rajasthan, India in 1996. Data are from Harris, D. and Bidinger, F. R. (unpublished).

6.   Cotton

Murungu et al. (2004a) tested priming of cotton seeds in on-station trials over 2 years at Save Valley Research Station, Zimbabwe, and in contrast to Murungu et al. (2003), reported that final emergence was reduced by priming in both the 1999–2000 and 2000–2001 seasons. Priming reduced final yield ($p < 0.05$) slightly, by 7%, in 1999–2000 and increased it ($p < 0.1$) by about 5% in 2000–2001, although both crops were thinned to a target population so it is not possible to disaggregate the effect of population density from other possible effects of priming.

7.   Mungbean

Priming mungbean for 8 h was tested in 15 irrigated on-station trials and four sets of rainfed, paired plot, farmer-participatory trials over four con-trasting years from 1999 to 2002 in the North West Frontier Province (NWFP) of Pakistan (Rashid et al., 2004b). Of the 19 trials, priming was

significantly better than nonpriming in 14 trials with a mean yield increase of 56%. In the remaining six trials there was no significant difference between treatments, but in no case was priming worse than not priming. Components of yield were measured in 11 of the trials. Priming sometimes increased slightly the number of plants m^{-2}, but in most cases, yield benefits were associated with better individual plant performance. Priming significantly increased pod length, number of pods per plant, and number of grains per pod. Total dry matter, shoot mass, and grain mass were all significantly increased by priming, although harvest index was not significantly affected, which suggests that overall growth was stimulated rather than just improved partitioning to reproductive structures.

8. Finger Millet

Priming seeds of six cultivars of finger millet with water for 8 h resulted in taller, earlier-maturing plants that produced more yield than plants from nonprimed seed in 2000 and 2001 (Kumar *et al.*, 2002). In 2000, priming significantly ($p < 0.05$) reduced both the mean time to 50% flowering and the mean time to maturity by about 6 days, increased mean plant height by 9 cm, and resulted in 17% extra grain. Priming increased grain yield significantly by 11% in 2001 although one variety (KARRA-1) failed to respond to priming.

9. Lentil

Neupane (2001) tested the effect of priming lentil seeds on growth and yield in two trials in Nepalgunj, Nepal in 1998–1999 and 1999–2000. Soaking seeds for 12 h, followed by drying in the shade for 2 h, was the best combination and it significantly ($p < 0.05$) reduced the number of days for 50% emergence from 9.5 to 8.2 days in 1998–1999 and from 9.3 to 7.4 days in 1999–2000. Although stand density was increased, on average, by about 16%, the effect was not statistically significant. Grain yield was significantly ($p < 0.05$) increased by 31% in 1998–1999 and by 37% in 1999–2000.

D. ON-FARM STUDIES

Chivasa *et al.* (1998) reported that farmers in semiarid Zimbabwe were well aware of the benefits of sowing seeds that had been soaked in water beforehand. The practice was particularly prevalent when gaps in poorly

established maize stands needed to be filled. Farmers knew that primed seeds germinated faster, and seedlings grew more rapidly than those established using nonprimed seed, thus helping plants in gaps to "catch up" with the rest of the crop. Farmers in marginal areas of western India also reported widespread knowledge of the practice of soaking chickpea seeds in water before sowing (Harris *et al.*, 1999). Hobbs *et al.* (1996) reported that farmers in the *Terai* of Nepal use a practice called *lewa* in which they soak rice seeds in water for 24 h before sowing into nurseries for subsequent transplanting. If sowing is postponed for any reason the primed seeds are dried in the shade for 1 day and then in the sun for another day. Treated in this fashion, seed can retain its viability for up to 1 month.

1. Wheat

Harris *et al.* (2001b) summarized data from 275 on-farm trials across South Asia from the lowland *Terai* region of Nepal, through Bihar, West Bengal, and Gujarat in India to the NWFP of Pakistan. All were simple, paired-plot trials, comparing nonprimed seed with seed primed "overnight" in water, and implemented by farmers on their own land using their own levels of management. Yields varied widely, from around 1.2–1.4 t ha^{-1} in Bihar and West Bengal in India and in Pakistan to about 2.3 t ha^{-1} in Nepal and about 4.2 t ha^{-1} in Gujarat. Priming increased grain yield at all sites and the extra grain production, averaged over all sites, was about 270 kg ha^{-1} (range 152–505 kg ha^{-1}). Percentage increases varied from 5% in Gujarat to 36% in the marginal, slightly saline area in Pakistan and were inversely proportional to the yield potential of the site but the absolute increases remained more constant. Woodruff (1973) tested priming and redrying wheat in Australia and concluded that effects were unlikely at yield levels above 2 t ha^{-1}. Harris *et al.* (2001b) speculated that seed priming might be compensating to some extent for low levels of management or for harsh environmental conditions that would otherwise limit yield. They concluded that "on-farm" seed priming was effective for wheat grown across a wide range of environments and gains were achieved at little or no cost to farmers. Feedback from farmers in Gujarat during participatory evaluation of the trials showed high rates of acceptance and adoption of seed priming.

In Pakistan, three series of on-farm trials implemented by 31 farmers over 3 years showed mean yield benefits due to priming for 16–18 h of 40%, 57%, and 20% (Harris *et al.*, 2004a). In contrast, also in Pakistan, two sets of participatory trials involving 13 farmers at Gundheri and 10 farmers at Mardan showed no significant response to priming (Rashid *et al.*, 2002).

2. Upland (Direct-Seeded) Rice

Three hundred and fifty-one farmers tested on-farm priming of upland rice using simple, paired-plot trials in western India between 1995 and 1998 (Harris *et al.*, 1999, 2001a). Direct measurements of yield were not made by researchers but detailed feedback from farmers was recorded and quantified during field days, farm walks, semistructured focus group discussions, and matrix ranking exercises (CARE, 1989; King, 2000). Farmers reported that both cereals, maize and rice, grown during the *kharif* (rainy season) had responded positively, and in a similar fashion, to priming overnight (farmers had ignored suggestions to soak seeds of both these crops for longer). The consensus view of farmers was that primed rice emerged earlier, by 1–3 days, and resulted in better and more uniform crop stands. Resowing, which carries a financial penalty and reduces yields because of the delay involved, was less common when crops were primed. Primed seedlings grew faster and more vigorously and sometimes flowered earlier. Uniform, vigorous stands competed better with weeds, and some farmers reported that less weeding was required. Some farmers, encouraged by the early growth, used fertilizer that they would not otherwise risk doing. Most farmers reported earlier maturity, sometimes up to 10 days, of primed crops and estimated higher yields. Ninety-five percent of a subsample of 56 farmers interviewed in 1996 indicated that they would prime their seeds the following year. Some farmers reported that primed seed, in contrast to nonprimed or pregerminated seed, sank immediately when sown into fields with free-standing water. Saikia *et al.* (1989) have noted that floating seeds can often drift or be blown by the wind before sinking and can result in clumping of seedlings and, ultimately, reduced yield.

Farmers in this area of western India try to grow a second crop in the *rabi* (postrainy season) after rice and the earlier harvest of rice, made possible by priming, was particularly appreciated because the second crop (e.g., wheat, chickpea, maize) could be sown earlier (Harris *et al.*, 2001a). Yields of *rabi* crops generally decline as sowing is delayed. For instance, yields of wheat in northern India are reduced by about 0.8% day^{-1} (about 100 kg ha^{-1}) for every day's delay in sowing after mid-November (Ortiz-Monasterio *et al.*, 1994). Witcombe and Harris (1997) have discussed the potential to exploit genetic- and priming-induced earliness to maximize total system productivity. Earlier harvest of *rabi* crops, apart from the yield advantages, has other benefits in that it allows earlier seasonal migration (and thus improved earning opportunities) from these marginal areas (Harris *et al.*, 2001a).

Overnight priming of seeds of cv. Kalinga III increased yield in all seven paired-plot trials implemented by farmers in Gujarat, India, with a mean increase of 23% (Harris *et al.*, 2001a). Farmers' opinions on seed priming agreed with those reported by Harris *et al.* (1999), that is, primed crops

emerged faster and more completely, produced more vigorous seedlings, flowered and matured earlier, and gave higher yields.

Upland rice is an important crop for resource-poor farmers in West Africa (WARDA, 2005), where the major constraints are drought and weed infestation (Adesina *et al.*, 1994). In Cameroon in 2000, 52 farmers primed rice seeds in water for 24 h then dried them for a further 24 h before sowing (WARDA, 2002, pp. 11–14). Comparisons with nonprimed crops were made during farm walks and open-ended discussions. Farmers reported that priming led to faster, better, more uniform establishment, more tillers, earlier flowering and maturity, and higher (average increase around 40%) grain yield. They also reported that primed crops tolerated dry spells better. Some farmers volunteered that, based on the evidence from their trials, they had primed their own rice and had also tried it successfully with maize and soybean. Fifteen farmers in the Gambia tested seed priming and reported earlier emergence, by about 2 days, but there were no yield differences associated with priming (WARDA, 2002, pp. 19–20).

Priming rice seeds for 12 h was tested in Ghana in 2000 by 30 farmers in Ejisu-Juabeng district (forest zone) and 20 farmers in the Ejura-Sekyeredumase district (forest-transition zone) (WARDA, 2002, pp. 21–29). Based on the on-farm trials, farmers reported a similar range of benefits to those reported from Cameroon (WARDA, 2002, pp. 11–14), including the perception that primed crops had greater tolerance to drought, and to (unspecified) pests and diseases. Mean yield increases due to priming for 12 h were 31% in Ejisu-Juabeng and 40% in Ejura-Sekyeredumase. Most farmers in Ejura-Sekyeredumase experienced some difficulties in sowing primed seed because they neglected to surface dry it before sowing, but this was easily remedied. There was spontaneous adoption of priming by farmers for their own, nontrial, crops where these had not already been sown and most intended to prime their rice seeds the following year.

One hundred farmers in Sierra Leone tested the priming response of two rice varieties (ROK 3 and a popular ''local'') in two locations (Lokomassama and Newton). Farmers' opinions concerning priming for 12 h were canvassed during 10 field days in 2000 (WARDA, 2002, pp. 52–56), and they reported benefits, including faster, more uniform emergence, more vigorous early growth, better competition with weeds, earlier maturity, and higher yields. Priming increased grain yield of ROK 3 by 42% and the local variety by 38% at Lokomassama and by 37% and 33%, respectively, at Newton.

Harris (2003) summarized results from 1937 farmers' trials of priming upland rice seeds in five West African countries between 1999 and 2002, including those trials reported in WARDA (2002). The mean yield increase due to priming in 657 trials in Ghana was 57% and in Nigeria from 440 trials it was 77%. In Cameroon, 421 farmers harvested, on average, 39% more rice

in primed plots and there were mean increases of 33% from 274 trials in Sierra Leone and 16% from 145 trials in The Gambia. Priming was particularly effective when there was drought, for example, in Ghana in 2001 the mean grain yield in nonprimed plots was only 0.53 t ha^{-1} whereas primed crops yielded 1.0 t ha^{-1}. In 20 of the 132 trials only the primed crop gave any yield. Only 8 trials of the 132 gave no benefit from priming, that is, a success rate of 94%, and there was widespread acceptance of priming among farmers who had tried it.

3. Maize

Fifty-three farmers tested maize-seed priming in the *kharif* (rainy) season in 1996, and 44 farmers tested it as a *rabi* (postrainy) season crop in 1996–1997 in tribal areas of Rajasthan, Gujarat, and Madhya Pradesh, India (Harris *et al.*, 1999). Farmers reported in pre- and postharvest focus group discussions that primed crops emerged 2–3 days earlier than nonprimed ones and resulted in better, more uniform stands (e.g., 95% vs 60% and 95–98% vs 70–75% were quoted) in both *kharif* and *rabi* seasons. Seventy-five percent of respondents in the *kharif* and 46% in the *rabi* initially encountered difficulties in sowing the (slightly swollen) primed seed because it got stuck in the tube (*pora*) that they used to direct seed into the furrow behind the plough. This was quickly remedied, however, by regulating more carefully the flow of seed into the *pora*. The local practice of mixing seeds with small amounts of granular diammonium phosphate was accommodated successfully by ensuring that the surface of primed seeds was dried thoroughly before mixing. Almost all farmers thought that primed crops grew more vigorously (and better competition with weeds was mentioned but not quantified), flowered and matured earlier, and produced bigger cobs and higher yield. Independent measurements on a subset of 35 trials showed a mean increase in cob weight of 6% (Harris *et al.*, 2001a). Farmers in the *kharif* were equivocal about whether primed crops tolerated dry spells better but 89% were of that opinion in the (drier) *rabi* season. Almost 100% of farmers intended to continue priming in the future.

A similar, participatory exercise with 51 farmers in four villages in semi-arid Zimbabwe also concluded that maize primed for 12 h emerged faster and more completely, but farmers were less emphatic about primed crops resisting dry spells or competing more effectively with weeds. The exercise was limited somewhat as only a few of the trials flowered and produced cobs in what was an extremely dry season, but it was generally held that primed crops flowered and produced cobs earlier (Harris *et al.*, 2001a). Harris *et al.* (2002a) reported results from a study in farmers' fields in Mushagashe and Zimutu in Zimbabwe where 38% of respondents indicated that they already

primed maize seeds, although generally for gap-filling rather than for initial sowings, a situation similar to that reported by Chivasa *et al*. (1998). Priming increased grain yield by an average of 14% consistently in three varieties grown in two contrasting years. Farmers noted earlier and more complete emergence and thus less gap-filling was required, which saved time and money. They also suggested that primed crops competed better with weeds. This could not be confirmed in on-station experiments to test this issue, although priming did increase plant height during the seedling stage, a characteristic sometimes associated with more competitive plants. Jasi *et al*. (2000) calculated the economics of priming and concluded that, primarily because of its low cost, there were net benefits.

4. Sorghum

Forty farmers primed sorghum seed in Musikavanhu communal area in Zimbabwe during the 1997–1998 season (Harris *et al*., 2001a). Although more than half misunderstood the need to surface-dry the wet seed and reported difficulties in handling it, most farmers agreed that priming accelerated emergence and plants flowered and matured earlier relative to nonprimed crops. In contrast, most farmers did not think that primed crops withstood drought spells better or smothered weeds to any great extent. Ninety-seven percent of participants intended to prime sorghum the following year. In 1998–1999, 171 farmers in the same area tested priming and described a similar range of benefits to the previous year (Harris, unpublished data). Yields, as measured by the farmers, were reported to be an average of 27% higher in primed plots but this could not be confirmed by independent measurement.

5. Chickpea

One hundred and one farmers in 17 villages in Rajasthan, Gujarat, and Madhya Pradesh states in India tested priming in chickpea during the 1995–1996 and 1996–1997 *rabi* seasons. Despite some initial difficulties in sowing seed primed for 8 h, almost all farmers reported earlier emergence (by 2–3 days) and better, more uniform stands that withstood drought better than nonprimed crops (Harris *et al*., 1999). Priming accelerated flowering (by 7–10 days) and the formation of pods. Crops matured earlier, by about 7–10 days with as much as 15 days reported in one case, and yields were higher. Independent corroboration by researchers of 10 trials in Bar, Gujarat and 8 trials in Bihar village, Madhya Pradesh showed mean advances in maturity of 7.6 and 6.7 days, respectively, while yield increases

due to priming were 45% in Bar and 15% in Bihar village. Earliness is valued in this area because of the prevalence of end-of-season drought that is particularly severe for *rabi* crops that often rely on residual moisture in the soil after the harvest of *kharif* crops (Joshi and Witcombe, 1996).

Musa *et al.* (2001) facilitated farmers in the High Barind Tract (HBT) of Bangladesh to test the effects of priming chickpea seeds sown after the harvest of *aman* (rainy season) rice. Rainfall averages 1285–1400 mm per year in this region but is concentrated during a single rainy season, supplementary irrigation is scarce, a second crop, such as chickpea, is difficult to establish properly (Mazid *et al.*, 1998) and yields are low (Rahman *et al.*, 1995). In 30 trials in the 1998–1999 season, priming chickpea seeds for 8 h increased yield significantly ($p < 0.05$) by 47% while, in a randomly-chosen subset of 35 of the 99 trials completed in 1999–2000, yields were significantly increased ($p < 0.05$) by 20%. Farmers reported a significant ($p < 0.001$) mean increase of 22% from the remaining 64 trials (Musa *et al.*, 2001). Yield also increased significantly ($p < 0.01$) by a mean of 17% in 15 researcher-managed demonstration trials using a different variety. Detailed measurements of emergence, growth, and yield components in both years showed that, averaged over the two strongly contrasting seasons, emergence, early growth, and plant stand at harvest were increased by about 21%, height at harvest by 10%, the number of pods m^{-2} by 25%, 1000-grain mass by about 5%, stover yield by 21%, and grain yield by 33%. Root nodules per plant were counted in 1999–2000 and primed plants had 48% more nodules ($p < 0.001$) than did nonprimed plants. Using data from all 144 trials, Musa *et al.* (2001) calculated the probability of farmers achieving a given yield with, and without, priming. For target yields above 0.5 t ha^{-1} priming increased the probability of success by 18% (for 0.75 t ha^{-1}), 18% (for 1 t ha^{-1}), 74% (for 1.5 t ha^{-1}), and 240% (for 2 t ha^{-1}). Musa (2000) noted that priming chickpea was already being widely adopted in the Barind, a conclusion supported by Saha (2002). Kumar Rao *et al.* (2004, 2005) have summarized the role of seed priming in the successful promotion of rainfed *rabi* cropping with farmers in remote areas of eastern India and Nepal.

6. Mungbean

Farmers' yields of mungbean were proportional to rainfall over four contrasting years in Pakistan (Rashid *et al.*, 2004b), and primed seed outperformed nonprimed seed in 34 out of 39 trials. Overall 4-year mean yield advantage due to priming was 33%, and there was no correlation between yield advantage and rainfall. Stand density in the 20 farmers' trials in 2002 was 37 plants m^{-2} in the nonprimed crops and 43 plants m^{-2} in the primed plots, an 18% increase ($p < 0.01$). Benefits from priming were the result of a

combination of faster germination and emergence and more vigorous growth and development, leading to better crop stands and bigger, more productive plants.

E. ADDED VALUE: IMPROVED CROP NUTRITION

1. Macronutrients

a. Nitrogen. Al-Mudaris and Jutzi (1999) primed sorghum and pearl millet seeds for 3 days at 25°C with a range of aerated solutions made up by dissolving commonly available fertilizers in water, followed by drying for 6 h. Although some solutions significantly increased rate of germination and final germination percentage relative to a control primed for 1 day in water, there were no treatment effects on seedling growth parameters measured 15 DAS and 60 DAS. Unfortunately, no nonprimed control was included and the soaking duration for the primed control was much longer than that reported as appropriate for sorghum by Harris (1991, 1996) and Chivasa *et al.* (2000) or for pearl millet by Harris and Mottram (2005).

Harris *et al.* (2001a) have reported that farmers have observed differences between primed and nonprimed crops in the color of the foliage, with the former sometimes having darker green canopies. This suggests that primed crops are taking up more nitrogen from the soil, perhaps as a consequence of more rapid early growth as noted by Dayanand *et al.* (1977) and Bhati and Rathore (1986) for wheat, particularly in relation to the changing availability of N in the seedbed around the time of sowing (Birch, 1960). Figure 3 shows the results from a trial in the Punjab, India, in which the response to added nitrogen fertilizer of wheat crops from primed- and nonprimed seeds was compared. Grain yield from primed crops was significantly greater than that from nonprimed crops at all four levels of added N. In practical terms, this offers the possibility of considerable savings on fertilizer costs since the same yield could be obtained from, for example, applying 50 kg ha^{-1} N and priming the seeds, as using 75 kg ha^{-1} without priming. Priming the seeds was the equivalent of saving 25 kg ha^{-1} N at application rates of 50, 75, and 100 kg N ha^{-1}. This improved nitrogen use efficiency is probably due to better uptake of N.

Harris *et al.* (2005a) reported that *Rhizobium* could be effectively added to the water used for priming chickpea seed—a finding also confirmed by Johansen (2004). Rupela *et al.* (1994) noted that resource-poor farmers are often reluctant to follow the recommended practice for applying *Rhizobium* inoculum, yet are happy to adopt on-farm seed priming (Saha, 2002). Thus, there is an opportunity to promote the application of *Rhizobium* through seed priming.
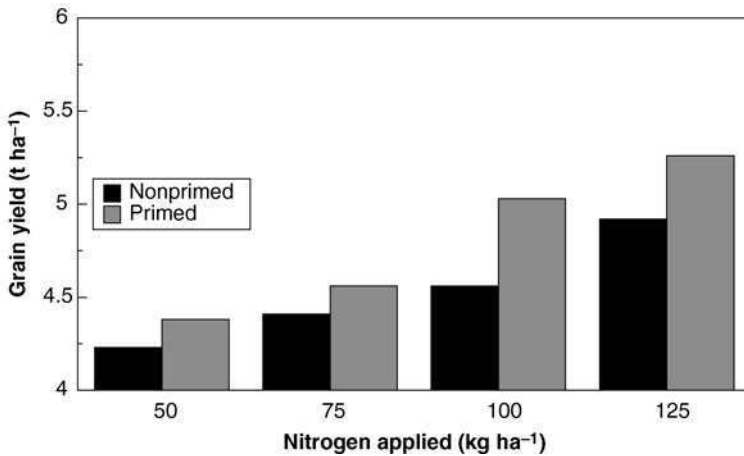
Figure 3 Grain yield of primed- and nonprimed wheat at four levels of applied nitrogen in Punjab, India. Data from Harris, D., and Malhi, S. S. (unpublished).

b. Phosphorus. Some authors have used priming with dilute phosphate solutions to promote uptake of P in wheat. For instance, Mujumdar and Somawanshi (1979) soaked seeds for 12 h in water or in various concentrations of KH_2PO_4 or NaH_2PO_4, but it is not recorded if seeds were subsequently dried. Phosphorus content of primed seeds increased as the concentration of the soaking solution increased, but priming with NaH_2PO_4 depressed seed potassium content. Priming with water alone increased seedling dry matter significantly ($p < 0.05$) relative to the nonprimed control at 7 DAS, 11 DAS, and 17 DAS, but P-content was only significantly increased at 7 DAS. In contrast, priming with 500 ppm P and 1000 ppm P delivered as KH_2PO_4 increased dry matter and P-content at all three dates over and above the increase associated with water-priming alone. However, there were no differences due to treatment in P-concentration of the shoots, implying that enhanced uptake was a consequence of faster growth and larger plants, consistent with the conclusions of DeMarco (1990). Singh and Chatterjee (1981) used Na_2HPO_4 (358 ppm for 6 h) to increase grain yield, relative to nonprimed seeds, by 19%, 29%, and 30% in trials in three consecutive years. However, averaged over the 3 years, this represents only an additional 8% advantage over that obtained from priming with water alone for 24 h.

Zhang *et al.* (1990) primed barley seeds for 24 h with 25.8 cM l^{-1} phosphorus as NaH_2PO_4 and recorded significant increases in seedling growth, particularly in seeds originally low in P. Seeds with high P-content took up less P and did not respond as well. The imbibed P was used by

seedlings as early as 3 DAS (Zhang *et al.*, 1998). Ajouri *et al.* (2004) showed that priming barley seeds for 12 h followed by redrying to 12% moisture content increased germination relative to nonprimed seeds from 65% to 95% and advanced germination by up to 3 days. The addition of 10 mM zinc (as $ZnSO_4$) and 50 mM phosphate (as KH_2PO_4) increased the P and Zn content of seeds, promoted uptake of P and Zn by seedlings and increased their water use efficiency under drought stress. Data in Table IV show a similar response to priming with KH_2PO_4 in maize, for which 1% w/v P is the optimum concentration and resulted in an advantage over and above that due to priming with water alone.

2. Micronutrients

a. Zinc. An FAO study (Sillanpa, 1982) on micronutrient status of the soils of Pakistan showed that about 62% of soils from Punjab, 100% from Sindh, and 7% from NWFP were deficient in Zn. Rashid (1996) considered zinc deficiency to be the third most serious crop nutrition problem in the country after N and P deficiency. Harris *et al.* (2005b) developed the priming technology that had been successfully demonstrated in Pakistan (Harris *et al.*, 2001b; Rashid *et al.*, 2002) to address the issue of zinc deficiency in wheat (Khattak and Parveen, 1986). Preliminary experiments with wheat had established that seeds could be primed safely with dilute solutions of ZnSO4 and that 0.4% w/v Zn was safe and effective. In eight on-station and on-farm trials between 2002 and 2004 crops from seeds primed for 10 h with 0.4% Zn as ZnSO4 produced a mean increase in grain yield of 615 kg ha^{-1} (21%) over crops from nonprimed seed. Data from two trials that included seed primed with water alone established that about half of this increase was due to zinc while half was due to the effect of priming with water.

Table IV
Effect of Priming Maize (cv. Kissan-90) with Water and Phosphate as KH_2PO_4

Variable	Treatment				Sig.	LSD (0.05)
	Not primed	Primed H_2O	Primed 1% w/v P	Primed 2%P		
Fresh shoot mass (g pl^{-1})	1.83[a]	2.04[a,b]	2.45[c]	2.17[b,c]	**	0.30
Dry shoot mass (mg pl^{-1})	186[a]	208[a,b]	242[b]	214[a,b]	*	36
Plant height (cm)	27.6[a]	29.5[a,b]	31.0[b]	28.9[a,b]	*	2.4

Data are from Rashid, A. and Harris, D. (unpublished). Variables were measured 14 DAS.

Benefit:cost ratio for soil application (calculated using data from two additional trials) was only 8, whereas for priming seeds with 0.4% zinc it was about 360 or about 160 for the response to zinc alone.

In alkaline soils in Pakistan, Harris *et al.* (2005b) found that the safe, optimum concentration for priming chickpea seeds with zinc (as $ZnSO_4$) was 0.05% Zn, much less than the optimum dose (0.4% zinc) for wheat. In nine trials sown in 2002 and 2003, this treatment increased grain yield of chickpea from 1050 to 1552 kg ha^{-1} in comparison with nonprimed seed. Yield increases in individual trials ranged from 10 to 122%, with a mean of 48%. Data from two trials that included seed primed with water alone established that about half of this increase was due to zinc, while half was due to the effect of priming with water. Benefit: cost ratio for priming chickpea with 0.05% Zn was 1500 or about 750 for the response to the zinc alone.

b. Molybdenum. Johansen *et al.* (2005) established that lack of molybdenum was limiting chickpea yields in many parts of the HBT of Bangladesh and that adding sodium molybdate to the soil increased grain yield by 73% in 2001–2002 and by 173%, 61%, and 58% at 3 sites in 2002–2003. However, no compound fertilizers containing Mo are available in Bangladesh, and it is impractical to evenly broadcast the small amount of Mo required (<500 g Mo ha^{-1}). Johnson (2004) showed that it was possible to use seed priming to increase safely the micronutrient content of chickpea seeds, including Mo and Zn. In a pot study using a moderately acidic soil of pH 6.1, priming for 8 h with 0.5 g l–1 sodium molybdate solution increased chickpea grain yield by 27% in comparison with Mo applied to the soil (Kumar Rao *et al.*, 2004). Priming also increased the Mo concentration of shoots from 0.21 to 6.94 ppm, roots from 0.4 to 12.92 ppm, and grain from 3.22 to 5.6 ppm.

Johansen *et al.* (2005) compared the effects of adding either Mo alone (0.5 g l^{-1}) or Mo with *Rhizobium* to the priming water with application of Mo to the soil surface. In three trials at three sites in the 2003–2004 season in Bangladesh, adding Mo alone to the priming water did not significantly improve yield over the control (seed primed with water alone but no Mo added to the soil), although adding both Mo and *Rhizobium* during priming increased mean grain yield by 51%, similar to the extra 55% grain obtained when Mo was applied directly to the soil.

Rice fallows—lands left fallow after harvesting rainfed rice—are widespread throughout South Asia with 2.1 M ha of rice fallows in Bangladesh, 0.4 M ha in Nepal, and 11.6 M ha in India (Subbarao *et al.*, 2001). The simple technologies developed to facilitate double cropping using chickpea in the Barind area of Bangladesh (Musa *et al.*, 2001) were tested in five States in eastern India where rice fallows are common. Johansen *et al.* (2005) tested the response of chickpea to Mo, either applied through priming or broadcast on the soil surface, and with inoculation of *Rhizobium* through priming,

in on-farm trials conducted on rice fallow lands with acid soils in eastern
India in 2003–2004. In 29 trials (spread over Orissa, Chattisgarh, eastern
Madhya Pradesh, Jharkhand, and West Bengal states) with chickpea cv.
ICCV 2, the mean yield increase over a control without Mo (mean yield 0.87
t ha^{-1}) was 22% when Mo was applied through seed priming water, and 20%
when Mo was applied to soil. In 19 trials with chickpea cv. KAK 2, the mean
yield increase over a control without Mo (mean yield 0.78 t ha^{-1}) was 17%
when Mo was applied through seed priming water and 25% when Mo was
applied to soil. The amount of Mo used when applied during seed priming is
much less, and more easily applied, than Mo added to the soil.

Khanal *et al.* (2005) in Nepal reported that adding 0.5 g l^{-1} sodium
molybdate to the priming water for chickpea significantly ($p < 0.01$)
increased the number of nodules per plant from 28 to 37 (32%) and the
grain yield from 0.45 t ha^{-1} to 0.54 t ha^{-1} (20%).

F. Added Value: Increased Pest and Disease Resistance

There have been several reports from the field that primed crops suffer
less from disease. Damage to chickpea plants in Bangladesh, due to soil-
borne diseases mainly caused by collar rot (*Sclerotium rolfsii*) and *Fusarium*
wilt, was significantly ($p < 0.001$) reduced by priming seeds overnight, by
45% in 1998–1999 and by 30% in 1999–2000 (Musa *et al.*, 2001). Harris *et al.*
(1999) reported Indian farmers' comments that primed chickpea suffered less
damage from pod borers, but the authors were unable to confirm this effect
in India; pod borer damage on chickpea in Bangladesh was much reduced in
primed crops, but the apparent difference was not statistically significant
(Musa *et al.*, 2001).

In a trial in Pakistan in 2002, Rashid *et al.* (2004a) reported that priming
seeds of mungbean cv. NM 92 for 8 h in water resulted in a significant
($p < 0.01$) fivefold increase in grain yield relative to a nonprimed crop. This
was associated with a large difference in the severity of symptoms of mung-
bean yellow mosaic virus (MYMV) assessed using a visual scoring index.
More than 70% of the nonprimed plants had severe or lethal symptoms
whereas only 14% of the primed plants were similarly affected. Only 9% of
nonprimed plants showed no disease symptoms in contrast to 32% of primed
plants. However, it was not possible to determine which of several possible
mechanisms were responsible for this large effect. Rashid *et al.* (2004b) also
observed similar differences in MYMV infection in other mungbean priming
trials.

Downy mildew disease, caused by the obligate biotroph *Sclerospora
graminicola* (Sacc.) Schroet. is a major constraint to pearl millet yields
(Jeger *et al.*, 1998; Singh *et al.*, 1993). A standard varietal screening method

(Jones *et al.*, 1995) was used to investigate the effect of seed priming on the disease resistance of pearl millet. Priming seeds in water for 8 h before sowing significantly reduced the incidence of downy mildew disease in seedlings of a highly susceptible cultivar from about 80% to less than 60% (Harris *et al.*, 2005a). Although the screen would not allow plants to be assessed at later stages of growth, there is a high degree of correlation between performance of cultivars in the screen and their resistance to downy mildew in the field (Jones *et al.*, 2002).

Many authors, for example, Sticher *et al.* (1997) and Métraux (2001) have reported instances of the phenomenon known as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (van Loon *et al.*, 1998) in a range of crops. SAR is a systemic enhancement of plant resistance to disease following a localized challenge by an elicitor which can be a microorganism, a chemical or even a physical stress. It is possible that the anaerobic stress (Blokhina *et al.*, 2003) associated with ''on-farm'' seed priming might induce SAR and thus explain the results of Harris *et al.* (2005a).

V. CONCLUSIONS

It is difficult to overemphasize the importance of rapid seedling emergence in marginal tropical environments. The promotion of rapid emergence, by whatever means, increases the probability of achieving good crop stands. Harris (1996) showed that delayed emergence can reduce subsequent relative growth rate of seedlings and, in general, healthy plants with well-developed root systems could withstand adverse conditions better than plants whose development and early growth have been interrupted at an early stage. Austin (1989) and Carter *et al.* (1992) reported that once a crop is established vigorous early growth is often associated with better yields. For pearl millet, Mohamed (1984) reported a good correlation between rate of germination and rate of canopy formation among genotypes, although he did not find a similar relation among groundnut genotypes.

In contrast, delayed emergence exposes seedlings to greater risk of damage due to high temperature, infection by soil-borne diseases, declining soil moisture, and physical impedance by hardening soils and the formation of soil crusts. Most studies reviewed here have shown that fast emergence is promoted by soaking seeds in water before sowing. This may (Al-Soqueer, 2004; Bhati and Rathore, 1986; Chivasa *et al.*, 2000) or may not (Neupane, 2001) result in differences in final emergence, but there are few examples where priming reduced emergence (Murungu *et al.*, 2004a, for cotton). Yield increases due to priming are not always only due to increased stand density (Murungu *et al.*, 2004b) but may be a combination of population density

and improved individual plant performance as reported by Musa *et al.*
(2001) for chickpea, Harris *et al.* (2002a) and WARDA (2002, pp. 21–29)
for rice, Rashid *et al.* (2004b) for mungbean, and Al-Soqueer (2004) for
sorghum.

In almost all cases, whenever priming resulted in rapid emergence, it also
promoted seedling vigor, and resulted in increased yield. Yields of primed
crops were almost never lower than those of nonprimed crops.

Most published work on seed priming involves drying seed to its original
water content. Studies in controlled environments (Al-Soqueer, 2004) that
have contrasted dried with nondried seed show that nondried seeds have a
clear advantage. However, it seems likely that few seeds primed by farmers
are sown immediately and thus many of the results of studies with dried
seeds may have relevance to the real world. In the case of maize, Finch-
Savage *et al.* (2004) even recommend aeration (difficult to achieve in the
field) or a drying delay of 1–2 h to allow full expression of the priming effect
in hot, wet conditions.

Resource-poor farmers were enthusiastic about priming, not just because
of the reduced risk of failure (Harris, 2003; Musa *et al.*, 2001) and increased
yield but also because primed crops flowered and matured earlier (Harris
et al., 1999, 2001a; WARDA, 2002) allowing more flexibility in their liveli-
hood strategies, for example, facilitating earlier sowing of following crops or
earlier migration to find off-farm work.

Higher yields from primed crops have, in a few cases, been associated
with measured reductions in damage from pests and diseases (Musa *et al.*,
2001; Rashid *et al.*, 2004a), and it may be that increased disease resistance
due to priming may contribute often to better crop performance undetected
where disease pressure is relatively low and few symptoms are apparent.
Certainly the *in vitro* work (Harris *et al.*, 2005a) suggests that may be the
case and is likely to be a fruitful field of study in the future.

Small amounts of water are always available, even in the remotest areas,
to prime crops and priming can, per se, improve nutrient use efficiency
(Fig. 3). Although it is technically possible, and highly cost effective, for
resource-poor farmers to use priming to supply some nutrients when they are
limiting production (Harris *et al.*, 2005b; Johansen *et al.*, 2005), opportu-
nities for them to do so will depend on improving access to less common
materials such as zinc sulfate or sodium molybdate. This is where crop
science meets community development and Kumar Rao *et al.* (2005) have
summarized work with development-oriented NGOs in eastern India to
empower farmer groups to bulk buy and distribute such materials.

Many of the major crops grown in tropical areas of developing countries
can benefit substantially from "on-farm" seed priming. It is a low-cost, low-
risk technology, is good insurance, and is readily adopted by farmers, once
they have tested it for themselves. The farmer-participatory trials reviewed

here have been critical in fine-tuning priming and promoting its adoption by resource-poor, risk-averse farmers. It is, in fact, particularly appropriate for small-scale farmers because priming is easier to accomplish with the relatively small amounts of seed they sow.

REFERENCES

Adesina, A. A., Johnson, D. E., and Heinrichs, E. A. (1994). Rice pests in the Ivory Coast West Africa: Farmers' perceptions and management studies. *Intl. J. Pest Manage.* **40**, 293–299.

Agrawal, R. P., Jhorar, B. S., Maiti, R. K., Raju, P. S., and Peacock, J. M. (1986). Effect of soil crusting on seedling emergence in sorghum genotypes. *Intl. J. Trop. Agric.* **4**(1), 15–22.

Agronomy Institute. (1982). Annual Report Winter 1979, Department of Research and Specialist Services, Ministry of Agriculture, Zimbabwe, p. 99.

Ajouri, A., Asgedom, H., and Becker, M. (2004). Seed priming enhances germination and seedling growth of barley under conditions of P and Zn deficiency. *J. Plant Nutr. Soil Sci.* **167**, 630–636.

Alessi, J., and Power, J. F. (1971). Corn emergence in relation to soil temperature and seeding depth. *Agron. J.* **63**, 717–719.

Al-Mudaris, M. A., and Jutzi, S. C. (1999). The influence of fertilizer-based seed priming treatments on emergence and seedling growth of *Sorghum bicolor* and *Pennisetum glaucum* in pot trials under greenhouse conditions. *J. Agron. Crop Sci.* **182**, 135–141.

Al-Soqueer, A. A. (2004). The potential of seed soaking in sorghum (*Sorghum bicolor* L. Moench) production. (Ph.D. Thesis). University of Nottingham, UK.

Amjad, M., Anjum, M. A., and Akhtar, N. (2004). Influence of phosphorus and potassium supply to the mother plant on seed yield, quality and vigour in pea (*Pisum sativum* L.). *Asian J. Plant Sci.* **3**(1), 108–113.

Ashraf, M., and Foolad, M. R. (2006). Pre-sowing seed treatment: A shotgun approach to improve germination, plant growth and crop yield under saline and non-saline conditions. *Adv. Agron.* **88**, 223–271.

Austin, R. B. (1972). Effects of environment before harvesting on viability. *In* "Viability of Seeds" (E. H. Roberts, Ed.), pp. 114–149. Chapman and Hall, London.

Austin, R. B. (1989). Maximising crop production in water-limited environments. *In* "Drought Resistance in Cereals" (F. W. G. Baker, Ed.), pp. 13–26. CAB International.

Bhati, D. S., and Rathore, S. S. (1986). Effect of seed soaking treatment with agro-chemicals on germination and seedling attributes of wheat. *Madras Agric. J.* **73**(7), 378–380.

Birch, H. F. (1960). Nitrification in soils after different periods of dryness. *Pl. Soil* **12**(1), 81–96.

Blokhina, O., Virolainen, E., and Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann. Bot.* **91**, 179–194.

Blum, A., and Sullivan, C. Y. (1986). The comparative drought resistance of landraces of sorghum and millet from dry and humid regions. *Ann. Bot.* **57**, 835–846.

Brar, G. S., Steiner, J. L., Unger, P. W., and Prihar, S. S. (1992). Modelling sorghum seedling establishment from soil wetness and temperature of drying seed zones. *Agron. J.* **84**, 905–910.

Brocklehurst, P. A., Dearman, J., and Drew, R. K. L. (1984). Effects of osmotic priming on seed germination and seedling growth in leek. *Hort. Sci.* **24**, 201–210.

Buckle, J. A., and Grant, P. M. (1974). Effects of soil temperatures on plumule growth and seedling emergence of maize (*Zea mays* L.). *Rhodesian J. Agric. Res.* **12**, 149.

CARE. (1989). Conducting Focus Group Interviews. Evaluation Unit, Program Department, CARE India.

Carter, D. C., Harris, D., Youngquist, J. B., and Persaud, N. (1992). Soil properties, crop water use and cereal yields in Botswana after addition of mulch and manure. *Field Crops Res.* **30**, 97–109.

CDS. (1990). Report on the KRIBHCO Rainfed Farming Project, Vol. 2: Annexes Centre for Development Studies, University of Wales, Swansea, UK.

Chastain, T. G., Ward, K. J., and Wysocki, D. J. (1995). Stand establishment responses of soft white winter wheat to seedbed residue and seed size. *Crop Sci.* **35**, 213–218.

Chhina, B. S., and Phul, P. S. (1982). Association of seed size and seedling vigour with various morphological traits in pearl millet. *Seed Sci. Technol.* **10**, 541–545.

Chiduza, C. (1987). On-farm evaluation of sorghum (*Sorghum bicolor* L.) varieties in the Sebungwe Region of Zimbabwe. (M.Phil. thesis), p. 140. Department of Soil Science and Agricultural Engineering, University of Zimbabwe.

Chiduza, C. (1993). An agronomic evaluation of ratooning and plant spacing in sorghum and comparative performance with maize and pearl millet in the northern Sebungwe, Zimbabwe. (D.Phil. thesis), p. 310. Department of Crop Science, University of Zimbabwe.

Chiduza, C., Waddington, S. R., and Mariga, I. K. (1994). Grain yield and economic performance of experimental open-pollinated varieties and released hybrids of maize in a remote semi-arid area of Zimbabwe. *Zimbabwe J. Agric. Res.* **32**, 33–43.

Chiduza, C., Waddington, S. R., and Rukuni, M. (1995). Evaluation of sorghum technologies for smallholders in a semi-arid region of Zimbabwe (Part I): Production practices and development of an experimental agenda. *J. Appl. Sci. Southern Africa* **1**, 1–10.

Chivasa, W. (1995). Survey of sunflower production constraints and comparative performance with maize and sorghum in Matibi II communal area of Zimbabwe. BSc. Agric. Hons. project, Department of Crop Science, University of Zimbabwe, p. 75.

Chivasa, W., Harris, D., Chiduza, C., Mashingaidze, A. B., and Nyamudeza, P. (2000). Determination of optimum on-farm seed priming time for maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* [L.] Moench) for use to improve stand establishment in semi-arid agriculture. *Tanzanian J. Agric. Sci.* **3**(2), 103–112.

Chivasa, W., Harris, D., Chiduza, C., Nyamudeza, P., and Mashingaidze, A. B. (1998). Agronomic practices, major crops and farmers' perceptions of the importance of good stand establishment in Musikavanhu Communal Area, Zimbabwe. *J. Appl. Sci. Southern Africa* **4**(2), 109–125.

Cotton Research Institute. (1986). Annual Report 1984/1985. Department of Research and Specialist Services, Ministry of Agriculture, Zimbabwe, p. 190.

Cotton Research Institute. (1996). Annual Report 1993/1994. Department of Research and Specialist Services, Ministry of Agriculture, Zimbabwe, p. 256.

Cromwell, E. (1996). "Governments, Farmers and Seed in a Changing Africa." CAB International, Wallingford, UK.

Cromwell, E. (1997). Local level seed activities: Opportunities and challenges for regulatory frameworks. *In* "New Seed and Old Laws: Regulatory Reform and the Diversification of National Seed Systems" (R. Tripp, Ed.), pp. 214–230. Intermediate Technology Publications, London.

Dayanand, M. K., Singh, K. N., and Agrawal, K. N. (1977). Effect of varieties, soil covers, forms of nitrogen and seed soaking on the uptake of major nutrients (NPK) in late sown wheat. *Indian J. Agron.* **22**(2), 96–98.

DeMarco, D. G. (1990). Effect of seed weight, and seed phosphorus and nitrogen concentrations on the early growth of wheat seedlings. *Aust. J. Exp. Agric.* **30**, 545–549.

Dhingra, K. K., Gill, G. S., and Kaul, J. N. (1974). Agronomic studies on the late-sown wheat. *J. Res.* **11**(3), 262–268.

Ellis, R. H. (1989). The effects of differences in seed quality resulting from priming or deterioration on the relative growth rate of onion seedlings. *Acta Hort.* **253**, 203–211.

Ellis, R. H., Hong, T. D., and Roberts, E. H. (1990). Effect of moisture content and method of re-hydration on the susceptibility of pea seeds to imbibition damage. *Seed Sci. Technol.* **18**(1), 131–137.

Evenari, M. (1980). The history of germination research and the lesson it contains for today. *Israel J. Bot.* **29**, 4–21.

Fakorede, M. A. B. (1985). Response of maize to planting dates in a tropical rainforest location. *Exp. Agric.* **21**, 19–30.

FAOSTAT. (2005). http://faostat.fao.org/

Finch-Savage, W. E., Dent, K. C., and Clark, L. J. (2004). Soak conditions and temperature following sowing influence the response of maize (*Zea mays* L.) seeds to on-farm priming (pre-sowing seed soak). *Field Crops Res.* **90**, 361–374.

Gan, Y., Stobe, E. H., and Moes, J. (1992). Relative date of wheat seedling emergence and its impact on grain yield. *Crop Sci.* **32**, 1275–1281.

Gill, K. S., and Prihar, S. S. (1989). Seedling emergence from a two-layered seed zone: Seeding depth and position, crop species and initial soil moisture effects. *Seed Sci. Technol.* **17**, 73–82.

Grant, P. M., and Buckle, J. A. (1974). Physical causes of failure in maize seedling emergence. *Rhodesia Agric. J.* **74**, 153–157.

Gubels, G. H. (1975). Emergence, seedling growth and yield of sweet corn after pregermination at high temperature. *Can. J. Plant Sci.* **55**, 995–999.

Gurmu, M., and Naylor, R. E. L. (1991). Effects of low water availability on germination of two sorghum cultivars. *Seed Sci. Technol.* **19**, 373–383.

Harris, D. (1991). Seedbeds and crop establishment. *In* "Proceedings of the Second Annual Scientific Conference of the SADDC-Land and Water Management Research Programme," pp. 165–172. October 7–9, 1991, Mbabane, Swaziland.

Harris, D. (1996). The effects of manure, genotype, seed priming, depth and date of sowing on the emergence and early growth of *Sorghum bicolor* (L.) Moench in semi-arid Botswana. *Soil Till. Res.* **40**, 73–88.

Harris, D. (2003). Reducing risk and increasing yields from rainfed crops in Africa using 'on-farm' seed priming. *In* "Abstracts: Harnessing Crop Technologies to Alleviate Hunger and Poverty in Africa," pp. 87–88. Sixth Biennial Conference of the African Crop Science Society, Hilton Nairobi Hotel, Kenya, October 12–16.

Harris, D., and Jones, M. (1997). On-farm seed priming to accelerate germination in rainfed, dry-seeded rice. *Int. Rice Res. Notes* **22**(2), 30.

Harris, D., and Mottram, A. (2005). Practical hydration of seeds of tropical crops: 'On-farm' seed priming. *In* "Handbook of Seed Science and Technology" (A. S. Basra, Ed.), pp. 724–734. The Howarth Press, New York (in press).

Harris, D., Breese, W. A., and Kumar Rao, J. V. D. K. (2005a). The improvement of crop yield in marginal environments using 'on-farm' seed priming: Nodulation, nitrogen fixation and disease resistance. *Austr. J. Agric. Res.* **56**(11), 1211–1218.

Harris, D., Hamdi, Q., and Terry, A. C. (1987). Germination and emergence of *Sorghum bicolor* (L.): Genotypic and environmentally-induced variation in the response to temperature and depth of sowing. *Plant Cell Environ.* **10**, 501–508.

Harris, D., Joshi, A., Khan, P. A., Gothkar, P., and Sodhi, P. S. (1999). On-farm seed priming in semi-arid agriculture: Development and evaluation in maize (*Zea mays* L.), rice (*Oryza sativa*) and chickpea (*Cicer arietinum*) in India using participatory methods. *Exp. Agric.* **35**, 15–29.

Harris, D., Pathan, A. K., Gothkar, P., Joshi, A., Chivasa, W., and Nyamudeza, P. (2001a). On-farm seed priming: Using participatory methods to revive and refine a key technology. *Agric. Syst.* **69**(1–2), 151–164.

Harris, D., Raghuwanshi, B. S., Gangwar, J. S., Singh, S. C., Joshi, K. D., Rashid, A., and Hollington, P. A. (2001b). Participatory evaluation by farmers of 'on-farm' seed priming in wheat in India, Nepal and Pakistan. *Exp. Agric.* **37**(3), 403–415.

Harris, D., Rashid, A., Ali, S., and Hollington, P. A. (2004). 'On-farm' seed priming with maize in Pakistan. *In* "Proceedings of the 8th Asian Regional Maize Workshop: New Technologies for the New Millennium" (G. Srinivasan, P. H. Zaidi, B. M. Prasanna, F. Gonzalez, and K. Lesnik, Eds.), pp. 316–324. August 5–8, 2002, Bangkok, Thailand, Mexico, D.F.: CIMMYT.

Harris, D., Rashid, A., Arif, M., and Yunas, M. (2005b). Alleviating micronutrient deficiencies in alkaline soils of the North-West Frontier Province of Pakistan: On-farm seed priming with zinc in wheat and chickpea. *In* "Micronutrients in South and South East Asia" (P. Andersen, J. K. Tuladhar, K. B. Karki, and S. L. Maskey, Eds.), pp. 143–151. Kathmandu: ICIMOD.

Harris, D., Rashid, A., Hollington, P. A., Jasi, L., and Riches, C. (2002a). Prospects of improving maize yields with 'on-farm' seed priming. *In* "Sustainable Maize Production Systems for Nepal" (N. P. Rajbhandari, J. K. Ransom, K. Adikhari, and A. F. E. Palmer, Eds.), pp. 180–185. Proceedings of a Maize Symposium, December 3–5, 2001, Kathmandu, Nepal. Kathmandu: NARC and CIMMYT.

Harris, D., Tripathi, R. S., and Joshi, A. (2002b). 'On-farm' seed priming to improve crop establishment and yield in dry direct-seeded rice. *In* "Proceedings of the International Workshop on Direct Seeding in Asian Rice Systems: Strategic Research Issues and Opportunities" (S. Pandey, M. Mortimer, L. Wade, T. P. Tuong, K. Lopez, and B. Hardy, Eds.), pp. 231–240. January 25–28, 2000, Bangkok, Thailand. Los Banos (Philippines), International Rice Research Institute.

Hegarty, T. W. (1978). The physiology of seed hydration and dehydration, and the relation between water stress and the control of germination: A review. *Plant Cell Environ.* **1**, 101–119.

Heydecker, W., and Coolbear, P. (1977). Seed treatments for improved performance survey and attempted prognosis. *Seed Sci. Technol.* **5**, 353–425.

Hobbs, P. R., Harrington, L. W., Adhikary, C., Giri, G. S., Upadhyay, S. R., and Adhikary, B. (1996). "Wheat and Rice in the Nepal *Tarai*: Farm Resources and Production Practices in Rupandehi District." Mexico, DF, CIMMYT and Nepal Agricultural Research Council.

Howarth, C. J., Weltzien Ratunde, E., Bidinger, F. R., and Harris, D. (1997). Seedling survival of abiotic stress: Sorghum and pearl millet. *In* "Proceedings of the International Conference on Genetic Improvement of Sorghum and Pearl Millet," pp. 379–399. ICRISAT/INTSORMIL, September 22–27, 1996, Lubbock, Texas.

Hunt, D. (1977). Poverty and agricultural development policy in a semi-arid area of eastern Kenya. *Africa Environ. Special Report* **5**, 74–91.

ICRISAT. (1986). Annual Report 1985, pp. 83–85. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India.

ICRISAT. (1987). Annual Report 1986, pp. 70–71. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India.

Idris, M., and Aslam, M. (1975). The effect of soaking and drying seeds before planting on the germination and growth of *Triticum vulgare* under normal and saline conditions. *Can. J. Bot.* **53**, 1328–1332.

Jaffee, J. (1991). "The Balance between Public and Private Sector Activities in Seed Supply Systems." The World Bank, Washington, DC.

Jasi, L., Gatsi, T., Ellis-Jones, J., and Riches, C. (2000). Participatory paired-plot comparison of primed and non-primed maize seed in Zimutu and Mushagashe. *In* "The Role of Small Dams in the Improvement of Rural Livelihoods in Semi-Arid Areas" (J. Ellis-Jones and V.

Zvarevashe, Eds.). CARE Stakeholder Workshop, Report IDG/00/18, Silsoe Research Institute, Bedford, UK.

Jeger, M. J., Gilijamse, E., Bock, C. H., and Frinking, H. D. (1998). The epidemiology, variability and control of the downy mildews of pearl millet and sorghum, with particular reference to Africa. *Plant Pathol.* **47**, 544–569.

Johansen, C., Musa, A. M., Kumar Rao, J. V. D. K., Harris, D., Ali, M. Y., and Lauren, J. G. (2005). Molybdenum response of chickpea in the High Barind Tract of Bangladesh and in Eastern India. *In* "Micronutrients in South and South East Asia" (P. Andersen, J. K. Tuladhar, K. B. Karki, and S. L. Maskey, Eds.), pp. 143–151. Kathmandu: ICIMOD.

Johnson, S. (2004). Improving micronutrient nutrition of various crops in the rice-wheat system of Nepal: Enrichment of legumes with boron and exploration of zinc redox chemistry in paddy rice soils. (Ph.D. Dissertation), p. 165. Cornell University, Ithaca, NY.

Jones, E. S., Breese, W. A., Liu, C. J., Singh, S. D., Shaw, D. S., and Witcombe, J. R. (2002). Mapping quantitative trait loci for downy mildew resistance in pearl millet: Field and glasshouse screens detect the same QTL. *Crop Sci.* **42**, 1316–1323.

Jones, E. S., Liu, C. J., Gale, M. D., Hash, C. T., and Witcombe, J. R. (1995). Mapping quantitative trait loci for downy mildew resistance in pearl millet. *Theor. Appl. Genet.* **91**, 448–456.

Jones, M. P., Dingkuhn, M., Aluko, G. K., and Semon, M. (1997). Interspecific *Oryza sativa* L. X. *O. glaberrima* Steud. Progenies in upland rice improvement. *Euphytica* **92**, 237–246.

Joshi, A., and Witcombe, J. R. (1996). Farmer participatory crop improvement. II. Participatory varietal selection, a case study in India. *Exp. Agric.* **32**, 461–477.

Kailasanathan, K., Rao, G. G. S. N., and Sinha, S. K. (1976). Effects of temperature on the partitioning of seed reserves in cowpea and sorghum. *Indian J. Plant Physiol.* **19**, 171–177.

Khan, A. A., Tao, K. L., Knypl, J. S., Borkowska, B., and Powell, L. E. (1978). Osmotic conditioning of seeds: Physiological biochemical changes. *Acta Hort.* **83**, 267–278.

Khan, A. A. (1992). Preplant physiological seed conditioning. *In* "Horticultural Reviews" (J. Janick, Ed.), pp. 131–181. John Wiley and Sons, New York.

Khanal, N., Joshi, D., Harris, D., and Chand, S. D. (2005). Effect of micronutrient loading, soil application, and foliar sprays of organic extracts on grain legumes and vegetable crops under marginal farmers' conditions in Nepal. *In* "Micronutrients in South and South East Asia" (P. Andersen, J. K. Tuladhar, K. B. Karki, and S. L. Maskey, Eds.), Micronutrients in South and South East Asia, pp. 121–132. Kathmandu: ICIMOD.

Khattak, J. K., and Parveen, S. (1986). Micronutrient status of Pakistan soils and their role in crop production. *Bull. Soil Sci.* No. 3. Peshawar: NWFP Agricultural University, Peshawar, Pakistan.

King, A. (2000). A brief review of participatory tools and techniques for the conservation and use of plant genetic resources. *In* "Participatory Approaches to the Conservation and Use of Plant Genetic Resources" (E. Friis-Hansen and B. Sthapit, Eds.), pp. 27–43. International Plant Genetic Resources Institute, Rome, Italy.

Klaij, M. C., and Hoogmoed, W. B. (1993). Soil management for crop production in the West African Sahel. II. Emergence, establishment and yield of pearl millet. *Soil Till. Res.* **25**, 301–315.

Knapp, R. (1966). Effect of various temperatures on the germination of tropical and subtropical plants. *Angew. Bot.* **32**, 230–241.

Kumar, A., Gangwar, J. S., Prasad, S. C., and Harris, D. (2002). 'On-farm' seed priming increases yield of direct-sown finger millet (*Eleusine coracana*) in India. *Int. Sorg. Mill. Newsletter* **43**, 90–92.

Kumar Rao, J. V. D. K., Harris, D., Johansen, C., and Musa, A. M. (2004). Low cost provision of molybdenum (Mo) to chickpeas grown in acid soils. Proceedings of the

IFA International Symposium on Micronutrients, February 23–25, 2004, New Delhi, India, International Fertilizer Industry Association (www.fertilizer.org).

Kumar Rao, J. V. D. K., Harris, D., Joshi, K. D., Khanal, N., Johansen, C., and Musa, A. M. (2005). Promotion of rainfed *rabi* cropping in rice fallows of eastern India, Bangladesh, and Nepal: An overview. *In* "Policy and strategy for increasing income and food security through improved crop management of chickpea in rice fallows in Asia" (S. Pande, P. C. Stevenson, R. K. Neupane, and D. Grzywacz, Eds.), pp. 64–75. Summary of a NARC-ICRISAT-NRI Workshop, 252 pp. November 17–18, 2004, Kathmandu, Nepal. International Crops Research Institute for the Semi-Arid Tropics. Patancheru 502324, Andhra Pradesh, India.

Legesse, N., and Powell, A. A. (1992). Comparison of water uptake and imbibition damage in eleven cowpea cultivars. *Seed Sci. Technol.* **20**(1), 173–180.

Linnemann, A. R., and Azam-Ali, S. N. (1993). Bambara groundnut (*Vigna subterranea*). *In* "Underutilised Crops, series II. Pulses and Vegatables" (J. T. Williams, Ed.), pp. 13–58. Chapman and Hall, London.

Lomte, M. H., Kawarkhe, P. K., and Ateeque, M. (1994). Dry sowing for improving the stability and productivity of rainfed sorghum in deep Vertisol. *J. Maharashtra Agric. Univ.* **19**(2), 195–197.

Lowveld Research Stations Summer report. (1969). Department of Research and Specialist Services, p. 46. Ministry of Agriculture, Rhodesia.

Mabika, V. C. (1992). Germination and emergence of bambara groundnut (*Vigna subterranean* L. Verdc.) in relation to temperature and sowing depth. MSc thesis, University of Nottingham, UK.

Maiti, R. K. (1986). Effect of planting depth on seedling emergence in sorghum (*Sorghum bicolor* L. Moench). *Seed Sci. Technol.* **17**, 83–90.

Maiti, R. K., Agrawal, R. P., Johrar, B. S., Raju, P. S., and Peacock, J. M. (1986). Effect of soil crusting on seedling emergence in sorghum. *Int. J. Trop. Agric.* **4**, 15–22.

Maiti, R. K., and Moreno, L. S. (1995). Seed imbibition and drying as a technique in evaluating sorghum lines for adaptation to dry sowing in the Semi-Arid Tropics. *Exp. Agric.* **31**, 57–63.

Mandal, A. K., and Basu, R. N. (1987). Mid-term and presowing hydration-dehydration treatments for improved field performance of wheat. *Field Crops Res.* **15**, 259–265.

Martin, J. H., and Leonard, W. H. (1957). "Principles of Field Crop Production." Macmillan Co., NY.

Massawe, F. J., Collinson, S. T., Roberts, J. A., and Azam-Ali, S. N. (1999). Effect of pre-sowing hydration on germination, emergence and early seedling growth of bambara groundnut (*Vigna subterranea* L. Verdc.). *Seed Sci. Technol.* **27**, 893–905.

Matthews, S. (1980). Seed physiology. *In* "Crop, Seed and Soil Environment" (J. Davies and J. B. Page, Eds.), pp. 70–85. Ministry of Agriculture, Fisheries and Food, UK.

Mazid, M. A., Wade, L. J., Saleque, M. A., Sarkar, A. B. S., Mollah, M. I. U., Olea, A. B., Amarante, S. T., and McLaren, C. G. (1998). Nutrient management in rainfed lowland rice for the High Barind Tract of Bangladesh. *In* "Rainfed Lowland Rice: Advances in Nutrient Management Research" (J. K. Ladha, Ed.), pp. 217–227. International Rice Research Institute, Manila, Philippines.

Métraux, J. P. (2001). Systemic acquired resistance and salicylic acid: Current state of knowledge. *Eur. J. Plant Pathol.* **107**(1), 13–18.

Mohamed, H. A. (1984). Varietal differences in the temperature responses of germination and crop establishment. (PhD thesis). University of Nottingham, UK.

Monteith, J. L. (1979). Soil temperature and crop growth in the tropics. *In* "Soil Physical Properties and Crop Production in the Tropics" (R. Lal and D. J. Greenland, Eds.), pp. 249–262. Wiley-Interscience, New York.

Mujumdar, A. J., and Somawanshi, R. B. (1979). Effect of pre-sowing seed soaking on emergence, dry matter production and uptake of phosphorus by wheat (*Triticum sativum* L.). *J. Maharashtra Agric. Univ.* **4**(3), 259–261.

Murungu, F. S., Chiduza, C., Nyamugafata, P., Clark, L. J., and Whalley, W. R. (2004a). Effect of on-farm seed priming on emergence, growth and yield of cotton and maize in a semi-arid area of Zimbabwe. *Exp. Agric.* **40**, 23–36.

Murungu, F. S., Chiduza, C., Nyamugafata, P., Clark, L. J., and Whalley, W. R. (2004b). Effects of 'on-farm seed priming' on consecutive daily sowing occasions on the emergence and growth of maize in a semi-arid Zimbabwe. *Field Crops Res.* **89**, 49–57.

Murungu, F. S., Nyamugafata, P., Chiduza, C., Clark, L. J., and Whalley, W. R. (2003). Effects of seed priming, aggregate size, and soil matric potential on emergence of cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.). *Soil Till. Res.* **74**, 161–168.

Murungu, F. S., Nyamugafata, P., Chiduza, C., Clark, L. J., and Whalley, W. R. (2005). Effects of seed priming and water potential on germination of cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.) in laboratory assays. *S. Afr. J. Plant Soil* **22**(1), 64–70.

Musa, A. M. (2000). On-farm chickpea seed priming trials and demonstrations in the High Barind Tract of Bangladesh, 1999–2000. Research Report, People's Resource Oriented Voluntary Association, p. 29. 204/A Uposhahar, Sopura, Rajshahi, Bangladesh.

Musa, A. M., Harris, D., Johansen, C., and Kumar, J. (2001). Short duration chickpea to replace fallow after aman rice: The role of on-farm seed priming in the High Barind Tract of Bangladesh. *Exp. Agric.* **37**(4), 509–521.

Nabi, G., Mullins, C. E., Montemayor, M. B., and Akhtar, M. S. (2001). Germination and emergence of irrigated cotton in Pakistan in relation to sowing depth and physical properties of the seedbed. *Soil Till. Res.* **59**, 33–44.

Nambiar, P. T. C., and Srinivasa Rao, B. V. (1987). Effect of sowing depth on nodulation, nitrogen fixation, root and hypocotyl growth and yield in groundnut (*Arachis hypogaea*). *Exp. Agric.* **23**, 283–291.

Neupane, R. K. (2001). Effect of seed priming on the growth and yield of lentil var. Khajura Masuro 2. *In* "Proceedings of National Winter Crops Workshop: Grain Legumes" (R. K. Neupane, N. K. Yadav, and R. Darai, Eds.), pp. 59–62. Nepal Agricultural Research Council, Khumaltar, Nepal.

Ong, C. K., and Monteith, J. L. (1984). Response of pearl millet and temperature. *In* "Agrometeorology of Sorghum and Millet in Semi-Arid Tropics. Proceedings of the International Symposium," pp. 129–142. ICRISAT, Patancheru, AP 502324, India.

Onwueme, I. C., and Sinha, T. D. (1991). "Field Crop Production in Tropical Africa: Principles and Practice." CTA, Ede, Wageningen, The Netherlands.

Oosterhout van, S. A. M. (1996). Coping strategies of smallholder farmers with adverse weather conditions regarding seed deployment of small grain crops during the 1994/1995 cropping season in Zimbabwe, Vols. 1–3. SADC/GTZ, Harare, Zimbabwe.

Ortiz-Monasterio, I., Dhillon, S. S., and Fischer, R. A. (1994). Date of sowing effects on grain yield and yield components of irrigated spring wheat cultivars and relationships with radiation and temperature in Ludhiana, India. *Field Crops Res.* **37**, 169–184.

Osburn, R. M., and Schroth, M. N. (1989). Effect of osmopriming sugar beet seed on germination rate and incidence of *Pythium ultimum* damping-off. *Plant Disease* **73**, 21–24.

Pal, S., Tripp, R., and Janaiah, A. (2000). "Public-Private Interface and Information Flow in the Rice Seed System of Andhra Pradesh (India)." Policy paper 12. National Centre for Agricultural Economics and Policy Research, New Delhi, India.

Parashar, A., and Varma, S. K. (1988). Effect of presowing seed soaking in gibberellic acid, duration of soaking, different temperatures and their interaction on seed germination and early seedling growth of wheat under saline conditions. *Plant Physiol. Biochem.* **15**(2), 189–197.

Parera, C. A., and Cantliffe, D. J. (1994). Presowing seed priming. *Univ. Florida J. Ser. No. R-03271* 1109–1141.

Paul, S. R., and Choudhury, A. K. (1991). Effects of seed priming with potassium salts on growth and yield of wheat under rainfed conditions. *Ann. Agric. Res.* **12**, 415–418.

Peacock, J. M. (1979). Sorghum physiology and crop establishment studies. "Final Scientific Report Phase II, Volume 2 Dryland Farming Research Scheme (DLFRS)." Gaborone, Botswana: Government Printer.

Peacock, J. M. (1982). Response and tolerance of sorghum to temperature stress. *In* "Sorghum in the Eighties: Proceedings of the International Symposium on Sorghum," pp. 143–159. ICRISAT, Patancheru, AP, India.

Peacock, J. M., and Heinrich, G. M. (1984). Light and temperature responses in sorghum. *In* "Agrometeorology of Sorghum and Millet in the Semi-Arid Tropics. Proceedings of the Intenational Symposium," pp. 143–158. ICRISAT, India. Patancheru, India.

Peacock, J. M., Miller, W. B., Matsuda, K., and Robinson, D. L. (1990). Role of heat girdling in early seedling death of sorghum. *Crop Sci.* **30**, 138–143.

Radford, B. J. (1983). Sowing techniques: Effects on crop establishment. Australian Institute of Agricultural Science Occasional Publication. No. 7, pp. 35–47.

Radford, B. J., Wood, I. M., Beavis, C. H., Walsh, P. A., Vieritz, A. M., Hazard, W. J. L., Wade, L. J., Hughes, P. J., Robertson, L. N., Page, J. R., Wollin, A. S., and Spackman, G. B. (1989). A survey of the establishment of commercial sorghum and sunflower crops in the Central Highland of Queensland and analysis of the effects of level of and evenness of establishment on grain sorghum. Occasional Publication 43. Australian Institute of Agricultural Science, Brisbane.

Rahman, M. M., Aziz, M. A., Musa, A. M., and Kumar, J. (1995). Prospects of pulse crops in the rice-based cropping system in Bangladesh. *In* "Fragile Lives in Fragile Ecosystems." Proceedings of the International Rice Research Conference, February 13–17, 1995. P.O. Box 933, Manila, Philippines.

Rao, S. K. (1981). Influence of seed size on field germination, seedling vigour, yield and quality in self pollinated crops: A review. *Agric. Rev.* **2**(2), 95–101.

Rashid, A. (1996). Secondary- and micronutrients. *In* "Soil Science" (E. Bashir and R. Bantel, Eds.), p. 374. National Book Foundation, Islamabad, Pakistan.

Rashid, A., Harris, D., Hollington, P. A., and Ali, S. (2004a). On-farm seed priming reduces yield losses of mungbean (*Vigna radiata*) associated with mungbean yellow mosaic virus in the North West Frontier Province of Pakistan. *Crop Protect* **23**, 1119–1124.

Rashid, A., Harris, D., Hollington, P. A., and Khattak, R. A. (2002). On-farm seed priming: A key technology for improving the livelihoods of resource-poor farmers on saline lands. *In* "Prospects for Saline Agriculture" (R. Ahmad and K. A. Malik, Eds.), pp. 423–431. Kluwer Academic Publishers, The Netherlands.

Rashid, A., Harris, D., Hollington, P. A., and Rafiq, M. (2004b). Improving the yield of mungbean (*Vigna radiata*) in the North West Frontier Province of Pakistan using on-farm seed priming. *Exp. Agric.* **40**(2), 233–244.

Rowland, J., and Whiteman, P. (1993). Principles of dryland farming. *In* "Dryland Farming in Africa" (J. R. J. Rowland, Ed.), pp. 68–94. Macmillan Press, London.

Rowse, H. R. (1996). Drum priming: A non-osmotic method of seed priming. *Seed Sci. Technol.* **24**, 281–294.

Rupela, O. P., Kumar Rao, J. V. D. K., Wani, S. P., and Johansen, C. (Eds.) (1994). Linking biological nitrogen fixation research in Asia: Report of a meeting of the Asia Working Group on Biological Nitrogen Fixation in Legumes, p. 140. December 6–8, 1993, ICRISAT Asia Center, India. Patancheru, Andhra Pradesh, India: ICRISAT.

Saha, A. K. (2002). Impact assessment study for the DFID-funded project R7540 promotion of chickpea following rainfed rice in the Barind area of Bangladesh CAZS natural resources, University of Wales, Bangor, UK.

Saikia, L., Pathak, A. K., and Baruah, B. P. (1989). Yield of rice sown in standing water. *Int. Rice Res. Notes* **14**(6), 16–17.

Sen, A., and Misra, N. M. (1984). Effect of pre-sowing treatment on the physio-morphological characters and yield of Kalyan Sona wheat under rainfed conditions. *Madras Agric. J.* **71**(3), 159–162.

Shumba, E. M., Bernstein, R. H., and Waddington, S. R. (1990). Maize and groundnut yield gap analysis for research priority setting in the smallholder sector of Zimbabwe. *Zimbabwe J. Agric. Res.* **28**(2), 105–113.

Siddique, K. H. M., and Loss, S. P. (1999). Studies on sowing depth for chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.) and lentil (*Lens culinaris* Medik) in a Mediterranean-type environment of South-western Australia. *J. Agron. Crop Sci.* **182**, 105–112.

Sillanpa, A. M. (1982). Micronutrients and nutrient status of soils. A global study. FAO Soil Bull. 48, Rome.

Sivakumar, M. V. K., Huda, A. K. S., and Virmani, S. M. (1984). Physical environment of sorghum- and millet-growing areas in South Asia. *In* "Agrometeorology of Sorghum and Millet in the Semi-Arid Tropics: Proceedings of the International Symposium," pp. 63–83. November 15–20, 1982, ICRISAT, Center, India.

Sivaprasad, B., and Sarma, K. S. S. (1987). Seedling emergence of chickpea (*Cicer arietinum* L.), pigeonpea (*Cajanus cajan* L.), and pearl millet (*Pennisetum typhoides* L.). Effect of differential soil crusting, as induced by raindrop size, and depth of sowing. *Plant Soil* **104**(2), 263–268.

Singh, A. I., and Chatterjee, B. N. (1981). Upland rice production with pre-treated seeds. *Indian J. Agric. Sci.* **51**(6), 393–402.

Singh, N. T., and Dhaliwal, G. S. (1972). Effect of soil temperature on seedling emergence in different crops. *Plant Soil* **37**, 441–444.

Singh, S. D., King, S. B., and Werder, J. (1993). Downy mildew disease of pearl millet. ICRISAT Information Bulletin No. 37. Patancheru, India.

Soman, P., Jayachandran, R., Bidinger, F. R., and Peacock, J. M. (1984a). Factors affecting seedling emergence and stand establishment: Studies in farmers' fields in Aurepally and Dhandhan during 1981, 1982 and 1983. Millet and Sorghum Physiology Program, Progress Report. ICRISAT, Patancheru, India.

Soman, P., Peacock, J. M., and Bidinger, F. R. (1984b). A field technique to screen seedling emergence of pearl millet and sorghum through soil crusts. *Exp. Agric.* **20**, 327–334.

Soman, P., Jayachandran, R., and Bidinger, F. R. (1987). Uneven variation in plant-to-plant spacing in pearl millet. *Agron. J.* **79**(5), 891–895.

Soman, P., Jayachandran, R., and Peacock, J. M. (1992). Effect of soil crusting on seedling growth in contrasting sorghum lines. *Exp. Agric.* **28**, 49–55.

Spencer, D. S. C., and Sivakumar, M. V. K. (1987). Pearl millet in African agriculture. *In* "Proceedings of the International Pearl Millet Workshop," pp. 19–31. April 7–11, 1986. ICRISAT Center, India.

Sticher, L., Mauch-Mani, B., and Métraux, J. P. (1997). Systemic acquired resistance. *Ann. Rev. Phytopathol.* **35**, 235–270.

Stroud, A. (1985). Finding solutions to tillage problems: A case study from the semi-arid areas of eastern province, Kenya. *In* "Proceedings of the Tenth East African Weed Science Society Conference," May 27–31, 1985. Department of Crop Science, University of Nairobi, P.O. Box 30197, Nairobi.

Subbarao, G. V., Kumar Rao, J. V. D., Kumar, J., Johansen, C., Deb, U. K., Ahmed, I., Krishna Rao, M. V., Venkataratnam, L., Hebbar, K. R., Sai, M. V. S. R., and Harris, D. (2001). Spatial distribution and quantification of rice-fallows in South Asia: Potential for legumes, p. 316. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, India.

Taylor, A. G., Klein, D. E., and Whitlow, T. H. (1988). Solid matrix priming of seeds. *Sci. Hort.* **37**, 1–11.

Taylor, A. G., Allen, P. S., Bennett, K. J., Burris, J. S., and Misra, M. K. (1998). Seed enhancements. *Seed Sci. Res.* **8**, 245–256.

Turner, M. R. (1994). Trends in India's seed sector. Paper presented at Asian Seed 94, September 27–29. Chiang Mai, Thailand.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopathol.* **36**, 453–483.

WARDA. (2002). Participatory Varietal Selection: Beyond the Flame (M. P. Jones and M. Wopereis-Pura, Eds.), p. 80. WARDA (West Africa Rice Development Association), Bouake, Cote d'Ivoire.

WARDA. (2005). Rice Trends in Sub-Saharan Africa, third ed., p. 34. WARDA (West Africa Rice Development Association), Bouake, Cote d'Ivoire.

Wilkinson, A. E. (1918). Soaking seeds before planting. *Market Growers J.* **22**, 6.

Willcocks, T. J., and Twomlow, S. J. (1993). A review of tillage methods and soil and water conservation in southern Africa. *Soil Till. Res.* **27**, 73–94.

Wilson, G. L., Raju, P. S., and Peacock, J. M. (1982). Effect of soil temperature on sorghum emergence. *Indian J. Agric. Sci.* **52**, 848–851.

Witcombe, J. R., and Harris, D. (1997). Impact of the DFID Plant Science Research Programme. Discussion Paper No. 3. Centre for Arid Zone Studies, University of Wales, Bangor, UK.

Woodruff, D. R. (1973). Evaluation of the pre-sowing drought hardening of wheat. *Queensland J. Agric. Anim. Sci.* **30**, 119–124.

Young, E., and Mottram, A. (2001). Transplanting sorghum and pearl millet as a means of increasing food security in semi-arid, low-income countries. *In* Tropical Agriculture Association Newsletter, December 2001, pp. 14–17.

Zhang, M., Nyborg, M., and McGill, W. B. (1990). Phosphorus concentration in barley (*Hordeum vulgare* L.) seeds: Influence on seedling growth and dry matter production. *Plant Soil* **122**, 79–83.

Zhang, M., Nyborg, M., and McGill, W. B. (1998). Phosphorus imbibed by barley seeds: Location within the seed and assimilation by seedlings. *Seed Sci. Technol.* **26**, 325–332.

Thermodynamic Modeling of Metal Adsorption onto Bacterial Cell Walls: Current Challenges

Jeremy B. Fein

Civil Engineering and Geological Sciences,
University of Notre Dame, Notre Dame, Indiana 46556

This chapter summarizes research that improves our understanding of metal-adsorption reactions involving bacterial cell walls. The chapter covers two general types of investigations: (1) those that aim to improve our molecular-scale understanding of metal adsorption onto bacteria, thereby improving the accuracy of thermodynamic bacterial adsorption models and (2) those that aim to improve our ability to apply thermodynamic models of metal-bacterial adsorption to complex realistic settings. The first type of research involves a range of experimental approaches, and the most common approaches are described here. The second type of research results from the need to balance the flexibility that comes with chemical sophistication in geochemical models with the practical impossibility of modeling the complexity of real systems at a molecular scale. Hybrid approaches that incorporate some degree of molecular-scale insight with testable simplifying assumptions are described and offer some hope for extrapolating our new-found molecular-scale understanding of metal–bacterial adsorption reactions to estimate mass transport in bacteria-bearing systems. The objective of this chapter is to describe both progress and remaining challenges in order to promote research that ultimately will yield accurate and flexible models of the effects of bacterial adsorption on metal distributions and speciation in natural geologic systems. © 2006, Elsevier Inc.

I.   INTRODUCTION

Bacteria are present in a wide range of low-temperature aqueous systems, and adsorption of aqueous metal cations onto bacterial cell walls can influence the speciation and mobility of metals in the environment (Bencheikh-Latmani *et al.*, 2003). Although the potential influence of bacterial adsorption of metals on geochemical processes has been realized for decades (Beveridge and Murray, 1976; Beveridge *et al.*, 1983), quantitative models of the effects of bacterial adsorption on metal distributions in water-rock systems still do not exist. Two major obstacles must be overcome in order to include bacterial adsorption effects in geochemical models of mass transport: (1) the molecular-scale mechanisms of metal adsorption onto bacterial cell walls must be better characterized so that accurate adsorption reactions can be formulated, and stability constants for the important metal–bacterial surface complexes can be determined and (2) commonalities between the adsorption behavior of different bacterial species must be ascertained. Although some constraints have been placed on bacterial adsorption mechanisms, the complexity of the cell wall and its associated structures make the task especially challenging. Because we know so little about adsorption mechanisms even for the relatively few species of bacteria that have been studied, it is not yet clear if adsorption mechanisms differ from species to species. The number of bacterial species of environmental and geologic interest is huge and undetermined, and if each species exhibits unique adsorption behavior, then it would be a nearly impossible task to develop quantitative models of bacterial adsorption in realistic systems.

The purpose of this chapter is to summarize the research that addresses these two obstacles and describe the avenues of research that seem most promising for overcoming these obstacles. The research that has been aimed at providing a molecular-scale understanding of bacterial cell wall adsorption includes studies from a wide range of experimental and modeling approaches. Each approach has strengths and limitations, and each approach provides a different type of constraint on the adsorption mechanisms and on the thermodynamic properties of the cell-wall surface complexes. There are relatively few studies that have addressed the challenges involved in extrapolating mechanistic investigations involving single bacterial species to quantifying bacterial adsorption of metals in complex realistic settings. This chapter summarizes a body of work that suggests that adsorption onto a wide range of bacterial species can be successfully modeled using a single set of stability constants for proton and metal-binding reactions. The study of adsorption mechanisms and metal adsorption in realistic settings is certainly far from complete, and this chapter is meant to assess our current state

of knowledge with the hope of inspiring research that will improve our understanding of these complex biogeochemical reactions even more.

The studies that are reviewed here involved nonmetabolizing bacterial cells only. These investigations not only serve as a baseline for studies of adsorption onto actively metabolizing cells but they also mimic the passive nonmetabolic adsorption that likely dominates in oligotrophic subsurface conditions. High rates of bacterial metabolism can be induced in engineered systems through the introduction of high concentrations of electron donors, and similar conditions can be found in some geologic settings. However, in general bacteria exist in the subsurface under nutrient-poor conditions (Ehrlich, 1996), and in these settings, passive cell wall adsorption will be significantly more important than adsorption onto cell walls of actively metabolizing cells.

II. MECHANISTIC STUDIES OF CELL WALL ADSORPTION

A. PARTITIONING RELATIONSHIPS VERSUS SURFACE COMPLEXATION MODELING

Metal adsorption onto bacterial surfaces has been studied extensively over the past 25 years. However, much of this previous work has been either qualitative or has quantified adsorption using a bulk-partitioning approach, making it impossible to estimate the effects of bacteria on mass transport under conditions different or more complex than those studied. Because of the complexities associated with natural systems, partitioning adsorption models are relatively simple to apply because they do not require a detailed understanding of the nature of the surfaces or adsorption mechanisms involved. That is, the extent of adsorption can be measured directly on a sample of material from the field, and a bulk partition coefficient can be determined, which describes the distribution of the species of interest between the bacterial surface and the other phase, or phases of interest. These are useful measurements, and they can be successful in describing adsorption/desorption processes for the site of interest. However, partition coefficients are applicable only to the conditions at which they were determined. Because the dominant adsorption reaction can change as a function of changing conditions, partition coefficient values can vary significantly as a function of pH, ionic strength, mineralogy, type of bacterial surface, aqueous solute speciation, and solute:surface area ratio (Bethke and Brady, 2000; Koretsky, 2000).

Over the past few years, experimental studies have demonstrated that surface complexation modeling can be used to account for metal adsorption onto bacterial surfaces (see Fein, 2000, for a review). This site-specific approach requires a thorough and comprehensive understanding of the cell wall-surface chemistry. Metal adsorption onto bacterial cell walls occurs because the cell walls contain a variety of organic functional groups, which display electrostatic and chemical affinities for positively charged metals. The most common organic functional groups present on the macromolecules present within bacterial cell walls are carboxyl, hydroxyl, and phosphoryl sites, with amino groups present to a lesser extent (Beveridge and Murray, 1980). Electrophoretic mobility data indicate that, typically, cell walls are uncharged at low pH (below approximately pH 2 in most cases), but they become increasingly negatively charged at higher pH values (Ams *et al.*, 2004; Claessens *et al.*, 2004; Fein *et al.*, 2005; Harden and Harris, 1953). Surface complexation modeling ascribes the electronegativity of the cell wall to deprotonation reactions involving cell wall organic functional groups.

$$R - AH^0 \Leftrightarrow R - A^{-1} + H^{+1} \tag{1}$$

where R represents the bacterial cell wall macromolecule to which each functional group type, A, is attached. Under this formalism, adsorption of aqueous metal cations (M^{m+}) onto deprotonated surface sites on the cell wall is represented as:

$$x(M^{m+}) + y(R - A^{-1}) \Leftrightarrow (R - A)_y(M)_x^{(xm-y)^+} \tag{2}$$
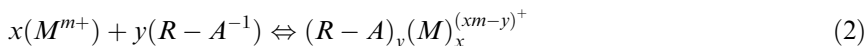
where x and y represent the stoichiometric coefficients and must be determined experimentally. Surface complexation modeling is so named because it explicitly accounts for the adsorbed metal as a "surface complex" or a distinct thermodynamic species with a fixed stoichiometry. The surface complex, like its cousin the aqueous complex, has a thermodynamic stability described by the equilibrium constant, K, which, for example, is here written for equilibrium (2):

$$k = \frac{[(R - A)_y(M)_x^{(xm-y)^+}]}{(a_{M^{m+}})^x[(R - A^{-1})]^y} \tag{3}$$

where brackets represent concentration in terms of moles of sites per kg of solution, and a represents the thermodynamic activity of the subscripted species.

Surface complexation models require a detailed understanding not only of the speciation of the aqueous species and surfaces involved but also of the adsorption/desorption mechanisms. A surface complexation model treats

the adsorbed metal as another species whose stability can be quantified with an equilibrium constant. By knowing the equilibrium constant values for each of the important equilibria in a system, the distribution of metals between various reservoirs (in solution, on mineral or bacterial surfaces) can be explicitly calculated. The equilibrium constants, which describe the extent of adsorption in surface complexation models, are invarient with respect to most of the parameters that affect partition coefficients. Therefore, the equilibrium constants determined in systems, which isolate specific adsorption reactions, can be combined in computational models of more complex systems. Modeling the effects of bacterial adsorption on mass transport in geologic settings using a surface complexation approach requires knowledge not only of the absolute concentrations of the dominant bacterial species but also a knowledge of the reaction stoichiometries and deprotonation and metal-binding constants for the functional group types on the bacterial cell walls.

B.   CONSTRAINTS ON BACTERIAL CELL WALL-PROTONATION REACTIONS

The choice of the most appropriate surface complexation model for cell wall adsorption is complicated by the complex chemical reactivity of bacterial surfaces. An aqueous organic acid, such as acetic acid, exhibits proton-buffering capacity over a relatively narrow pH range. This range is limited to the pH region in which significant concentrations of both the protonated and deprotonated species exist simultaneously, a pH range of 1–2 pH units to either side of the acidity constant for the acid. The organic acid functional groups on bacterial cell walls, however, exhibit an almost continuous buffering capacity over a wide pH range from, typically, approximately pH 2–10. There is considerable uncertainty whether this protonation/deprotonation behavior is best explained due to three to four functional group types that exhibit discrete pK_a values (Cox et al., 1999; Fein et al., 1997, 2005; Haas et al., 2001), or whether a smaller number of (one or two) functional groups with a distribution of pK_a values are responsible (Martinez et al., 2002; Plette et al., 1995). A range of experimental approaches has been used to constrain the nature of the protonation reactions on bacterial cell walls. Each approach has strengths and limitations, yet an unambiguous understanding of cell wall-protonation behavior remains elusive.

Similarly, there is considerable uncertainty concerning the best approach for accounting for the effects of the surface electric field associated with bacterial cell walls. Fein et al. (Daughney and Fein, 1998; Fein et al., 1997; Yee and Fein, 2001) have used an approach analogous to that applied to mineral surface electric fields, employing a constant capacitance model

(CCM) in conjunction with the Boltzmann equation (Stumm and Morgan, 1996). Plette *et al.* (1995, 1996), Martinez *et al.* (2002), and Yee *et al.* (2004b) utilize Donnan models, which unlike the constant capacitance model, do not assume a planar charge at the bacterial surface but rather assume a uniformly distributed charge throughout the cell wall volume. A number of models are possible because there are few data that constrain the properties of the electric field associated with bacterial cells. One approach that appears to be promising for probing the nature and extent of electrostatic interactions with cell walls is atomic force microscopy (AFM) (Camesano and Logan, 2000; Lower *et al.*, 2000). For example, Camesano and Logan (2000) used AFM measurements to demonstrate that electrostatic repulsion forces between bacterial polymers and the AFM tip are larger and extend over longer distances (up to hundreds of nanometers) than predicted by DLVO theory, and that pH effects dominate over ionic strength effects. These types of measurements are crucial in order to formulate a theory that explains the effects exerted by bacterial cell wall-electric fields on the distribution and speciation of protons and metals on the cell wall.

Most of the information on cell wall-protonation behavior has come from potentiometric titrations of suspensions of bacterial cells. In these experiments, the change in pH of a bacterial suspension is measured after additions of a known volume of acid or base. Essentially, these are proton-adsorption experiments that measure the concentration of free protons (solution pH) for a condition at which the total number of protons added to the system (the concentration of acid/base) is known. These measurements are powerful tools for determining unequivocally the total number of protons that adsorb onto bacterial cell walls. Although titration data yield rigorous constraints on total proton-active site concentrations on the cell wall, bacterial surface charge properties and mechanisms responsible for protonation behavior cannot be uniquely determined from potentiometric titrations alone (Fein *et al.*, 2005).

A growing number of potentiometric studies of the protonation of bacterial cell walls have been conducted (Cox *et al.*, 1999; Daughney and Fein, 1998; Fein *et al.*, 1997, 2005; Haas *et al.*, 2001; Martinez *et al.*, 2002; Ngwenya *et al.*, 2003; Plette *et al.*, 1995; Sokolov *et al.*, 2001; van der Wal *et al.*, 1997; Wightman *et al.*, 2001; Yee and Fein, 2001; Yee *et al.*, 2004b). These studies are consistent in that each indicates that functional groups on bacterial surfaces are proton active over a broad pH range, including down to pH values of as low as 2 and up to pH values as high as at least 10. Studies of protonation behavior of the cell wall-functional groups is complicated outside this pH range due to structural damage that is sustained by the cell wall when exposed to acidic or basic solution conditions (Borrok *et al.*, 2004c). The buffering intensity of the bacterial cell walls is characterized by two broad peaks of maximum buffering intensity: one at pH 4–5 and one above pH 8.5 (Fein *et al.*, 2005).

Despite the general agreement between data-sets that measure bacterial cell wall-protonation behavior with titrations, these studies apply a wide range of modeling approaches to interpret the potentiometric data. For example, Plette *et al.* (1995) used a three-site Langmuir–Freundlich model (LF), coupled with the Gibbs–Donnan shell model, to account for their experimental acidimetric titration measurements of *Rhodococcus erythropolis* A177. Fein *et al.* (1997) conducted potentiometric titrations of *Bacillus subtilis* and modeled the results with three discrete cell wall-functional groups, using a constant capacitance model. Cox *et al.* (1999), in similar experiments, but using a nonelectrostatic (neglecting the effects of the double layer) linear programming method modeling approach (Brassard *et al.*, 1990), modeled titration data using five discrete binding sites. Martinez *et al.* (2002) interpreted potentiometric titration data for *B. subtilis* and *Escherichia coli* by considering a set of equally spaced ($\Delta pK_a = 0.2$) pK_a values and using the Gibbs–Donnan shell model to account for electrostatic effects, finding evidence for four types of bacterial surface functional groups. Fein *et al.* (2005) demonstrated that a range of models, from a discrete site nonelectrostatic model (NEM) to a Langmuir–Freundlich continuous pK_a distribution approach, can equally well describe a single set of potentiometric titration data (Fig. 1). As the range of titration models that can be found in the literature demonstrates the buffering behavior, and intensity can be modeled either with a relatively large number (four) of discrete proton-active sites that are unaffected, or only minimally affected, by surface electric field effects, or with fewer sites that are proton active over a wider pH range than
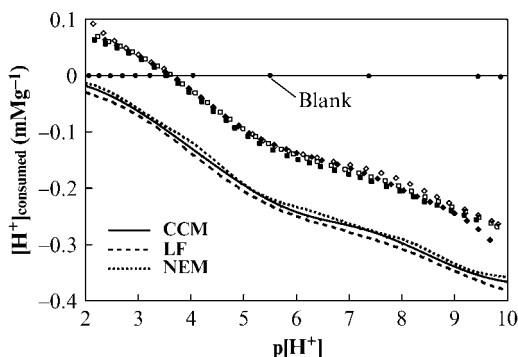
Figure 1 Potentiometric titration data of 75–150 g l^{-1} (wet weight) *B. subtilis* in 0.1 M NaClO$_4$. The data are calculated with [H$^+$]$_{consumed}$ = 0 at the pH of suspension. The model curves are calculated taking into account T_H^0, and hence are displaced downward by this amount. The three curves are from a CCM, a LF, and a NEM. Within error, each of these modeling approaches provides a reasonable fit to the experimental data. Reprinted from Fein *et al.* (2005). *Geochim. Cosmochim. Acta*, **69**, 1123–1132, with permission from Elsevier.

the discrete sites in the "nonelectrostatic" models. The wider pH range of influence can be caused either by the effects of a bacterial surface electric field, or it may be the result of "site" heterogeneity, or it may result from a combination of the two effects.

Additional constraints on the molecular-scale protonation mechanisms exhibited by bacterial cell walls have been provided by infrared spectroscopic investigations. Infrared spectroscopy has been used as a direct determination of the identity of bacterial functional group types, with studies demonstrating the importance of carboxyl, phosphoryl, hydroxyl, and amino groups (Benning *et al.*, 2004a,b; Dittrich and Sibler, 2005; Jiang *et al.*, 2004; Yee *et al.*, 2004a). Most of the studies use infrared spectroscopy as a qualitative tool to identify functional groups present on cell walls. However, Yee *et al.* (2004a) and Jiang *et al.* (2004) also use infrared spectroscopy to investigate the pH dependence of the protonation state of cell wall-functional groups. The number of pH conditions studied in each investigation is limited, but the coverage is sufficient to demonstrate an increase in the vibrational frequencies of deprotonated carboxyl groups with increasing pH. The ability to discern protonated from deprotonated functional groups using infrared spectroscopy suggests that more detailed examinations of the pH dependencies could possibly be used to provide constraints on the mechanisms of proton adsorption and to distinguish between discrete or continuously distributed acidity constants for functional group sites.

Calorimetric measurements of bacterial surface protonation reactions offer another relatively untapped resource for constraining proton adsorption mechanisms. Calorimetry measurements of proton adsorption onto bacterial cell walls not only can provide rigorous constraints on the extent of bulk proton adsorption but also interpretation of these data using a surface complexation modeling approach can yield site-specific enthalpies and entropies of proton adsorption onto the bacterial surface functional groups. These data can be interpreted to yield information on proton coordination environment, as well as the temperature dependence of the protonation reactions. Gorman-Lewis *et al.* (2006) have conducted the only calorimetric study of bacterial surface protonation to date. Their results (Fig. 2) indicate that the protonation reaction is exothermic, with calculated site-specific entropies of protonation that are relatively small. This suggests that the functional groups on the bacterial surface behave more like multifunctional organic acids with nearby proton-active sites rather than simple monofunctional acids with a single isolated functional group. Hydrogen bonding between protonated and deprotonated sites likely occurs as reflected by the relatively small entropies of protonation. The calorimetry results suggest that multifunctional organic acids likely represent better analogues than monofunctional acids for modeling the acidity behavior of the functional groups present on bacterial surfaces.
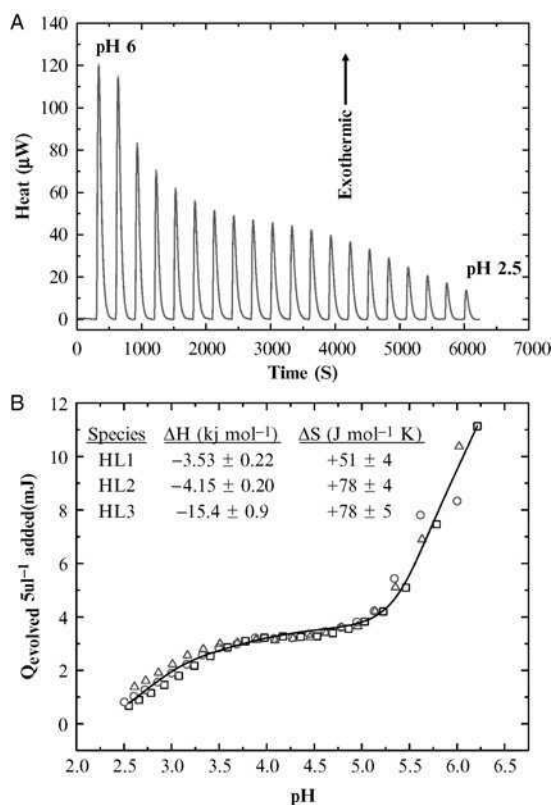
Figure 2 (A) Raw data from a typical low-pH proton adsorption calorimetric titration experiment, showing heat associated with adsorption of protons onto functional groups on the bacterial cell wall of *B. subtilis*. Data show continuous uptake of protons, and that the cell wall-functional groups are not fully protonated even under the lowest pH conditions of these experiments. (B) Corrected heat evolved from three low-pH proton adsorption calorimetric titration experiments involving *B. subtilis*, with the curve representing the best fit to the data assuming three types of protonation sites each with their own discrete pK_a value. Each figure indicates that protonation reactions are exothermic with heats comparable to those associated with protonation of multifunctional organic acids. The calculated site-specific enthalpies and entropies of protonation are listed in (B), and the low values for ΔS suggest hydrogen bonding between functional group sites. Reprinted from Gorman-Lewis *et al.* (2006, in press) *Geochim. Cosmochim. Acta*, with permission from Elsevier.

There are two main types of studies of bacterial surface protonation reactions: ones like potentiometric titration experiments that provide primarily bulk proton adsorption information and ones like infrared spectroscopy and calorimetry that yield information on proton adsorption mechanisms.

The first type of experiments provide rigorous constraints on total proton-active functional group site concentrations and the data are the easiest to use to calculate acidity constants and specific site concentrations for individual functional groups on the bacterial surface. However, as discussed earlier, titrations offer little information on molecular-scale protonation mechanisms. Conversely, infrared spectroscopy and calorimetry have the potential to significantly enhance our understanding of bacterial cell wall reactivity, but the signal can be difficult to interpret unequivocally, and the data are not geared toward constraining thermodynamic parameters of the bacterial surface species. Clearly, better constraints on mechanisms of protonation of bacterial cell wall-functional groups will come from a combination of these and other approaches.

C. Constraints on Mechanisms of Metal Adsorption onto Bacteria

Bulk metal-adsorption experiments, conducted as a function of pH and solute:sorbent ratio, provide constraints on the stoichiometries and thermodynamic stabilities of the important metal–bacterial surface species. A number of studies over the past decade have used surface-complexation modeling of bulk metal-adsorption measurements to determine binding mechanisms for metals onto bacterial cell walls (Fein *et al.*, 1997; Martinez and Ferris, 2001; Plette *et al.*, 1996). Bulk adsorption studies have demonstrated that metal and proton adsorption onto bacterial cell wall-functional groups typically is rapid and reversible (Daughney and Fein, 1998; Fowle and Fein, 2000). Furthermore, surface complexation modeling can successfully account for metal competition, pH effects, and changing solute:sorbent ratio on metal distributions in bacteria-bearing systems. Fowle and Fein (1999) demonstrated that stability constants for metal–bacterial surface complexes that are derived from single metal, single bacterial species experiments can be used to accurately estimate the extent of adsorption that occurs in more complex mixed systems. In addition, stability constants for bacterial surface complexes that involve metals that have not been studied directly can be estimated using linear free energy predictive approaches that quantify relationships between metal–bacterial stability constants and stability constants for metals complexed to aqueous organic acid anions (Fein *et al.*, 2001).

Although bulk adsorption experiments offer only circumstantial evidence of the dominant adsorption reactions, these measurements can provide constraints on the identity and thermodynamic stability of important metal–bacterial surface complexes. For example, Gorman-Lewis *et al.* (2005) measured aqueous uranium adsorption onto *B. subtilis* as functions of pH, solid:solute ratio, and dissolved CO_2 and Ca concentrations.

In oxygenated, CO_2-rich systems, negatively charged uranyl–carbonate complexes dominate the aqueous uranium speciation, and it is commonly assumed that these complexes exhibit negligible adsorption onto negatively charged surfaces such as bacteria. However, Gorman-Lewis et al. (2005) observed extensive uranium adsorption onto the bacterial surface, including under conditions where negatively charged aqueous uranyl–carbonate complexes dominate the aqueous uranium speciation (Fig. 3). Thermodynamic modeling of the data suggests that uranyl–hydroxide, uranyl–carbonate, and calcium–uranyl–carbonate species each can form stable surface complexes on the bacterial cell wall, results that could dramatically alter predictions of uranium mobility in near-surface environments. Similarly, Markai et al. (2003) interpreted bulk Eu(III) adsorption measurements using a surface complexation modeling approach, demonstrating that Eu(III) binds with both carboxyl and phosphoryl functional groups on B. subtilis bacterial cell walls. The adsorption data constrain both the reaction stoichiometry and the thermodynamic stabilities of the important Eu-bacterial surface complexes, parameters that are crucial for modeling the effects of bacterial adsorption on the mobility of Eu in bacteria-bearing geologic systems.

   Although bulk adsorption experiments represent a powerful approach for quantifying stability constants of metal-adsorption reactions, the data provide relatively weak circumstantial constraints on the molecular-scale mechanisms responsible for the metal adsorption. More rigorous constraints on adsorption mechanisms are offered by a range of complementary
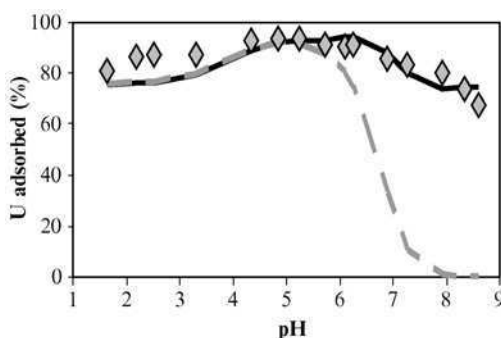
Figure 3 Measured adsorption of aqueous U(VI) onto B. subtilis bacterial cells (0.5 gm wet mass l^{-1}) suspended in solutions in equilibrium with atmospheric CO_2. Dashed curve represents a thermodynamic model prediction for the extent of adsorption assuming that uranyl–carbonate complexes do not adsorb and remain in solution; solid curve represents the best-fitting model constructed assuming that uranyl–carbonate bacterial surface complexes form. Data suggest that significant adsorption of aqueous uranyl–carbonate complexes onto the bacterial cell wall can control U speciation and mobility in near-neutral waters. Reprinted with permission from Gorman-Lewis et al. (2005). Copyright 2005, American Chemical Society.

experimental approaches, and a number of these approaches have been applied successfully to examine metal–bacterial adsorption reactions. X-ray absorption spectroscopy (XAS) has been used to constrain the binding environment for metal cations on cell wall-functional groups (Boyanov *et al.*, 2003; Francis *et al.*, 2004; Hennig *et al.*, 2001; Kelly *et al.*, 2002; Panak *et al.*, 2002a,b; Sarret *et al.*, 1998; Songkasiri *et al.*, 2002; Templeton *et al.*, 2003). Spectroscopic data provide more direct evidence of the metal-coordination environment than do bulk adsorption measurements. However, due to the complexity of the cell wall-binding environments, and due to the fact that XAS yields averaged information on all binding environments sampled by the technique, the experimental results from XAS can be complex and difficult to interpret. For example, although XAS has been used successfully to demonstrate the importance of carboxyl and phosphoryl binding sites for U and Cd on the bacterial cell wall of *B. subtilis*, significant uncertainties exist regarding the metal:ligand stoichiometry involved in binding (Boyanov *et al.*, 2003; Kelly *et al.*, 2002). Although the XAS data from these studies do not conclusively determine whether binding sites on bacterial cell walls exhibit discrete or continuous acidity constants, the data do require at least three distinct binding sites over the pH range of 3.5–7.0, and the data do not point to the type of binding complexity that might be expected for a cell wall on which functional groups deprotonate continuously over a wide pH range.

Calorimetric measurements of metal binding onto bacterial surfaces, interpreted using a surface complexation modeling approach, can be used to infer site-specific enthalpies and entropies of metal adsorption. Weppen and Hornburg (1995) used flow calorimetry to compare the metal sequestration capability of cell wall suspensions harvested from a range of bacterial cells. Their measured bulk enthalpies of metal adsorption (for Cd, Cu, Zn, Pb, Mg, Ca, Sr, and Ba) were all modestly endothermic ($+3$ to $+18$ kJmol^{-1}), and in the expected range for divalent cations coordinated with anionic oxygen ligands. However, Weppen and Hornburg (1995) did not correct for the heat associated with protonation reactions, and used a bulk-partitioning approach instead of a site-specific surface complexation model to quantify metal adsorption. Gorman-Lewis *et al.* (2006) used calorimetric data, in conjunction with surface complexation models of the protonation of the bacterial cell wall, to produce site-specific enthalpies and entropies of Cd adsorption onto the cell wall of *B. subtilis*. The calculated entahlpies of Cd adsorption are typical for Cd complexation with anionic oxygen ligands; the entropies are indicative of inner sphere complexation by multiple ligands; and the calorimetry results also constrain both the stoichiometry of the adsorption reactions and the temperature dependence of the stability constants for the dominant metal–bacterial surface complexes.

The need to elucidate mechanisms responsible for metal adsorption onto bacterial cell walls has led to the use of other molecular-scale experimental approaches. For example, time-resolved laser-induced fluorescence spectroscopy (TRLFS), like XAS, can characterize the binding environment of fluorescent elements attached to bacterial cell wall-functional groups. Texier *et al.* (2000) used TRLFS to determine that Eu binding onto *Pseudomonas aeruginosa*, a gram-negative bacterial species, occurs primarily through carboxyl and phosphoryl binding at pH 6. Similar results were obtained by Markai *et al.* (2003), who studied Eu adsorption onto the gram-positive species *B. subtilis* using TRLFS, and found carboxyl groups primarily responsible for Eu binding at pH 5, with phosphoryl groups becoming important at pH 7. The TRLFS data also provide some constraints on the metal:ligand ratio for the dominant adsorption reactions. In contrast to the mixed carboxyl and phosphoryl binding observed in the studies by Texier *et al.* (2000) and by Markai *et al.* (2003), Panak *et al.* (2000, 2002b) used TRLFS, in conjunction with EXAFS, to demonstrate that U(VI) forms inner sphere complexes only with phosphate groups on cell walls of a number of *Bacillus* species at pH 4.5–5.0.

Bulk adsorption measurements and a range of complementary experimental approaches have shed light on the molecular-scale mechanisms responsible for metal adsorption onto bacterial cell walls, however, considerably more work must be conducted to yield a complete understanding of these important geochemical reactions. Our understanding of the protonation behavior of cell wall-functional groups is rudimentary, and a range of protonation models can be invoked to explain observed protonation behaviors. Although spectroscopic and calorimetric investigations of metal binding onto bacterial cells can provide tighter constraints on the cell wall reactivity, there have been relatively few detailed studies to date. Due to the growing number of experimental and theoretical approaches, the next 5 years of research is likely to yield dramatically improved and detailed understandings of the mechanisms that control metal binding onto bacterial cell walls. One of the most important challenges that must be addressed is to better determine the nature and protonation behavior of the proton-active sites on the cell wall. Although potentiometric titrations offer rigorous constraints on total site concentrations, the data do not effectively distinguish between sites that exhibit discrete deprotonation behavior and sites that influence each other and exhibit relatively continuous deprotonation over a wide pH range. Furthermore, future research should include investigations into bacterial surface electric field effects and how to characterize them. Some attempts at this have been made using mineral surface electric field models. However, the three-dimensional nature of the cell wall and its constituent macromolecules suggests that a more sophisticated approach may be required (Wasserman and Felmy, 1998; Wasserman *et al.*, 2000). A number of

spectroscopic approaches have been used to study metal binding onto bacterial cell walls. Although much has been learned, our mechanistic understanding of these reactions is still rudimentary and considerably more research can be conducted in order to elucidate the dominant adsorption reaction stoichiometries, and hence binding environments, responsible for metal complexation on bacterial cell walls.

III. CHALLENGES IN APPLYING SURFACE COMPLEXATION MODELS TO REAL SYSTEMS

Laboratory and field studies have demonstrated that bacterial cell walls efficiently adsorb a variety of aqueous metal cations and organic molecules (Baughman and Paris, 1981; Beveridge and Murray, 1976, 1980; Gonçalves *et al.*, 1987; Harvey and Leckie, 1985; Konhauser *et al.*, 1993). Under some conditions, bacteria are mobile in the subsurface (Allen and Morrison, 1973; Bengtsson and Lindqvist, 1995; Gannon *et al.*, 1991; Harvey *et al.*, 1989, 1993; Johnson and Logan, 1996; McDowell-Boyer *et al.*, 1986), and they can enhance contaminant mobilities through adsorption of contaminants onto bacterial cell walls (Champ *et al.*, 1984; Champlin and Eichholz, 1976; McCarthy and Zachara, 1989). Bacteria can also be immobile due to biofilm formation, bacterial adsorption onto mineral surfaces, and/or due to bacterial straining by the rock matrix. When immobile, metal adsorption onto the bacteria is likely to enhance metal contaminant retardation (Malard *et al.*, 1994; Matthess and Pekdeger, 1985). Whether bacteria are mobile or immobile, when they represent a significant portion of the solid surface area with which groundwater comes into contact, bacteria-water-rock interactions must be quantitatively accounted for in order to accurately assess mass transport in realistic subsurface environments. Most experimental studies of metal adsorption onto bacterial cell walls involve only a single bacterial species. There are a number of challenges that must be addressed in order to extrapolate these findings from studies involving individual bacterial species to realistic settings that include a myriad of species.

One of the most problematic obstacles to the application of surface complexation modeling to realistic systems is the determination of adsorption site concentrations in geologically and biologically complex systems (Davis *et al.*, 1998; Payne *et al.*, 2004). A bacteria-bearing natural system can contain a large number of different bacterial species, and the number of species of environmental interest is huge and undetermined. If bacterial surfaces are unique and if each species exhibits unique adsorption properties, then it would be a Herculean task to determine the binding site concentrations and binding constants for each bacterial species of environmental interest.

Experimentation on dozens of different bacterial species would be necessary just to describe the metal behavior in a single environment. If bacterial species differ significantly from each other in their adsorptive behaviors, then the complexities of realistic systems would make it virtually impossible to model metal speciation and distribution on a molecular scale due to practical computational limitations. Conversely, application of too simple a model, which does not account for surface and solute speciation changes with changing subsurface conditions, can lead to inaccurate predictions of metal speciation and mobility (Bethke and Brady, 2000; Koretsky, 2000).

A potential solution to this dilemma arises from the observations that a number of bacterial species exhibit similar extents of metal adsorption (and similar proton and metal-binding constants) as determined in laboratory experiments using individual pure strains of bacteria (Daughney *et al.*, 1998; Kulczycki *et al.*, 2002; Ngwenya *et al.*, 2003; Small *et al.*, 1999). Yee and Fein (2001) hypothesized that a common mechanism of adsorption exists for a wide range of bacterial species, and they conducted potentiometric titrations and Cd–bacteria adsorption experiments using a range of gram-positive and two gram-negative bacterial species. Yee and Fein (2001) observed one common adsorption edge (Fig. 4 for the wide variety of bacterial species studied, suggesting that the structures that give rise to metal and proton adsorption onto bacteria are common over a wide range of bacterial species. Based on the universality of the Cd-adsorption edge, Yee
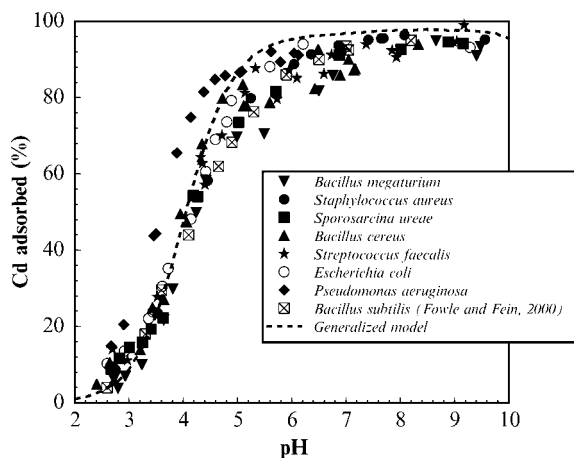
Figure 4 Cd adsorption onto pure cultures of individual gram-positive and gram-negative bacterial species. Each data point represents individual batch experiments with $10^{-4.1}$ M Cd and 1.0 g l^{-1} (dry wt.) bacteria. The dashed curve represents the adsorption behavior calculated using the average parameters given in Yee and Fein (2001). Reprinted from Yee and Fein (2001). *Geochim. Cosmochim. Acta* (2001) **65**, 2037–2042, with permission from Elsevier.

and Fein (2001) proposed that averaged thermodynamic parameters and site concentrations can be used to predict Cd adsorption onto bacterial surfaces for all bacterial species over a wide range of conditions, regardless of which species or groups of species are present in the system of interest.

The hypothesis of universal bacterial adsorption behavior has been supported by a number of subsequent experimental studies. Yee and Fein (2003) tested the universality of bacterial adsorption by measuring metal-adsorption behaviors of a mixture of 5 different gram-positive species, a mixture of 5 different gram-negative species, and a mixture of the 10 gram-positive and gram-negative bacteria, noting that all mixtures that were tested exhibited nearly identical extents of adsorption. Jiang *et al.* (2004) used attenuated total reflectance Fourier-transform infrared spectroscopy of both gram-positive and gram-negative bacterial species to demonstrate that the infrared spectra of both gram-positive and gram-negative bacteria are similar and exhibit similar variations as a function of changes in pH. The similarity in spectra for this range of bacteria suggests a similarity in binding environment for metals, and this observation supports a universal adsorption behavior that is rooted in similar cell wall-functional group chemistries. Borrok *et al.* (2004a) measured H^+ and Cd adsorption onto bacterial consortia from a range of natural environments. Their results indicate that the consortia adsorb similar extents of protons and Cd, and that the adsorption onto all of the consortia can be modeled using a single set of stability constants (Fig. 5.). In addition, Borrok *et al.* (2005) compiled all available potentiometric
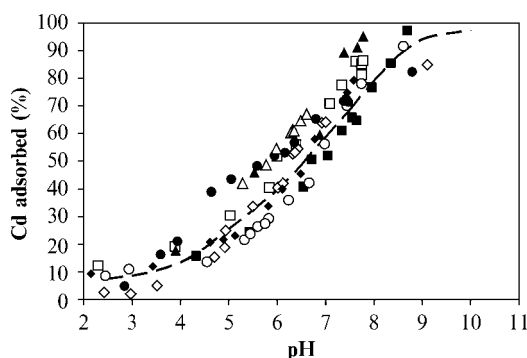
Figure 5 Data from Borrok *et al.* (2004a). Cd adsorption onto bacterial consortia cultured from soil and aquatic environments. All Cd adsorption experiments were conducted using 10 ppm Cd and 10 g l^{-1} consortia (wet weight) in a suspension of 0.1 M NaClO$_4$. The consortia were grown using soil broth (SB) or trypticase soy broth (TSB) with 0.5% yeast extract. ($\blacksquare$) = Forest soil #1, SB; ($\square$) = wastewater effluent, TSB; ($\blacktriangle$) = wastewater effluent, SB; ($\diamond$) = forest soil #2, SB; ($\circ$) = river water, SB; ($\bullet$) = wetland water, TSB; ($\blacklozenge$) = wetland soil, SB; and (Δ) = wetland water, SB. The dashed curve represents the best-fit adsorption edge that is calculated using the "universal" averaged thermodynamic parameters. Reprinted from Borrok *et al.* (2004a). *Geochim. Cosmochim. Acta* **68**, 3231–3238, with permission from Elsevier.

titration data-sets for individual bacterial species, bacterial consortia, and bacterial cell wall components. They note general similarities between proton adsorption behaviors of the range of bacterial species studied to date, and they use the data to construct an internally consistent surface complexation model for all suitable data-sets, presenting an averaged set of "universal" thermodynamic proton binding and site density parameters for modeling bacterial adsorption reactions in geologic systems. Modeling results demonstrate that the total concentrations of proton-active functional group sites for the 36 bacterial species and consortia tested are remarkably similar, averaging 3.24×10^{-4} moles per wet gram with a $1\,\sigma$ uncertainty of 1.0×10^{-4} moles per wet gram. Examination of the uncertainties involved in the development of proton-binding modeling parameters suggests that ignoring factors, such as bacterial speciation, ionic strength, temperature, and growth conditions, introduces relatively small error compared to the unavoidable uncertainty associated with the determination of cell abundances in realistic geologic systems. Hence, Borrok *et al.* (2005) proposed that reasonable estimates of the extent of bacterial cell wall deprotonation can be made using averaged thermodynamic modeling parameters, regardless of bacterial species used, ionic strength, temperature, or growth condition of the experiment.

Although a large number of bacterial species appear to exhibit broadly similar adsorption behavior, some studies suggest that at least some bacteria have unique adsorptive properties. For example, Borrok *et al.* (2004b) showed that bacteria that thrive in highly perturbed contaminated environments may exhibit significantly different adsorptive behavior from those from uncontaminated systems. Borrok *et al.* (2004b) measured proton and Cd adsorption onto a range of bacterial consortia grown from heavily contaminated industrial wastes, groundwater, and soils, and modeled the results using a discrete site surface complexation approach to determine binding constants and site densities for each consortium. The results demonstrate that bacterial consortia from different contaminated environments exhibit a range of total site densities (approximately a threefold difference) and Cd-binding constants (approximately a tenfold difference), and Borrok *et al.* (2004b) suggest that the range of adsorption behaviors is related to the evolutionary pressures on the bacteria in each of the contaminated environments.

IV. CONCLUDING REMARKS

The complexity and heterogeneity of realistic natural systems mean that modeling the transport and distribution of mass on a molecular scale is practically impossible due to computation limitations. Conversely, the application of too simple an adsorption model can lead to inaccurate

predictions of metal speciation and mobility in the subsurface (Bethke and Brady, 2000), and therefore models of contaminant transport must include some chemical complexity in order to account for the effects of changing conditions (pH, mineralogy, biomass concentration variability, and so on) on contaminant adsorption behavior. Clearly, a compromise approach must be determined in which sufficient chemical complexity in the form of individual adsorption site specificity for each bacterial and mineral surface is incorporated into geochemical models, but generalities are recognized to enable an approximate solution to be calculated. There is a clear tradeoff between the precision associated with calculated metal speciations and metal distributions and the ability to make such calculations. That is, when modeling metal mobility in the subsurface, generalized models with relatively high degrees of associated uncertainty may be acceptable, while for engineered systems, in which compositional heterogeneities and complexities are well-characterized, more precision is obtainable and a generalized model may be less appropriate than one that explicitly accounts for adsorption site complexities for the particular bacterial species of interest.

This chapter demonstrates that considerable progress has been made both in understanding the molecular-scale mechanisms of metal and proton adsorption onto bacterial cell wall-functional groups and in constructing generalized adsorption models that can be applied to complex systems. However, considerable work remains on both fronts. Mechanistic models of metal adsorption require better characterization of cell wall adsorption sites and adsorption reactions. We must answer questions as fundamental as whether cell wall-functional groups exhibit continuous or discrete deprotonation behavior, we must better constrain the number of types and concentrations of each functional group present on cell walls, and we have relatively poor constraints on the stoichiometries of metal-adsorption reactions. The research summarized in this chapter demonstrates the need to collect mechanistic information from a wide range of experimental approaches in order to rigorously constrain adsorption reaction mechanisms. The most effective approaches used to date include bulk adsorption experiments, a range of spectroscopies, and molecular modeling; however, other approaches are likely to offer new perspectives and should be pursued. Each approach has strengths and weaknesses associated with it, so each provides only a limited view of the overall picture.

In addition to the work required to better characterize metal-adsorption mechanisms on bacterial cell walls, more research is also required in order to understand the effects exerted on metal speciation by biological structures associated with bacteria in natural environments. For example, most bacteria in natural systems do not exist as discrete cells but are present within attached biofilms that are composed primarily of extracellular polymers that may significantly affect metal adsorption and speciation themselves.

While some research has focused on metal binding to these extracellular polymers and to biofilms in general (Acosta *et al.*, 2005; Freire-Nordi *et al.*, 2005; Lau *et al.*, 2005; Marques *et al.*, 1990; Toner *et al.*, 2005), these studies are in their infancy, and considerably more research is required in order to quantify the importance of polymer adsorption relative to cell wall adsorption.

The similar adsorption behavior that is exhibited by most single bacterial species and mixed bacterial consortia, which have been studied to date, suggests that metal-adsorption experiments conducted using only a limited number of types of bacteria (or perhaps even one type) can be extrapolated to reasonably accurately model metal adsorption onto complex mixed bacterial populations if the total concentration of bacteria is known. However, a number of challenges must be addressed before this type of modeling approach can be implemented with confidence. The biggest experimental challenges are to better understand the origin of the observed adsorption similarities and to determine the limits of the universal adsorption-behavior assumption.

Universal adsorption behavior by bacteria would eliminate the need to characterize absolute concentrations and distributions of individual bacterial species in a geologic system. However, the next challenge for application of bacterial-adsorption models to real systems would be to improve our methods for determining overall bacterial site concentrations in realistic settings. While this would be unquestionably more straightforward than mapping out distributions and concentrations of individual bacterial species, quantitative approaches have yet to be designed or tested. It is likely that stochastic-modeling approaches, similar to those used to characterize hydraulic and facies heterogeneities in groundwater aquifers (Huysmans and Dassargues, 2005; Moysey *et al.*, 2003), may prove fruitful.

Mechanistic studies of metal adsorption onto bacterial cell walls and research aimed at field-scale adsorption models are not mutually exclusive activities. The results from each type of study should help to guide the research in the other area. Continued sustained efforts on both fronts eventually will improve not only our molecular-scale understanding of bacterial adsorption reactions but also our ability to apply that understanding to model the effects of adsorption on mass transport in realistic geologic settings.

ACKNOWLEDGMENTS

REFERENCES

Acosta, M. P., Valdman, E., Leite, S. G. F., Battaglini, F., and Ruzal, S. M. (2005). Biosorption of copper by *Paenibacillus polymyxa* cells and their exopolysaccharide. *World J. Microbiol. Biotechnol.* **21**, 1157–1163.

Allen, M. J., and Morrison, S. M. (1973). Bacterial movement through fractured bedrock. *Ground Water* **11**, 6–10.

Ams, D. A., Fein, J. B., Dong, H., and Maurice, P. A. (2004). Experimental measurements of the adsorption of *Bacillus subtilis* and *Pseudomonas mendocina* onto Fe-oxyhydroxide-coated and uncoated quartz grains. *Geomicrobiol. J.* **21**, 511–519.

Baughman, G. L., and Paris, D. F. (1981). Microbial bioconcentration of organic pollutants from aquatic systems: A critical review. *Crit. Rev. Mircobiol.* **8**, 205–228.

Bencheikh-Latmani, R., Leckie, J. O., and Bargar, J. R. (2003). Fate of uranyl in a quaternary system composed of uranyl, citrate, goethite, and *Pseudomonas fluorescens*. *Environ. Sci. Technol.* **37**, 3555–3559.

Bengtsson, G., and Lindqvist, R. (1995). Transport of soil bacteria controlled by density-dependent sorption kinetics. *Water Resources Res.* **31**, 1247–1256.

Benning, L. G., Phoenix, V. R., Yee, N., and Tobin, M. J. (2004a). Molecular characterization of cyanobacterial silicification using synchrotron infrared micro-spectroscopy. *Geochim. Cosmochim. Acta* **68**, 729–741.

Benning, L. G., Phoenix, V. R., Yee, N., and Konhauser, K. O. (2004b). The dynamics of cyanobacterial silicification: An infrared micro-spectroscopic investigation. *Geochim. Cosmochim. Acta* **68**, 743–757.

Bethke, C. M., and Brady, P. V. (2000). How the Kd approach undermines ground water cleanup. *Ground Water* **38**, 435–443.

Beveridge, T. J., and Murray, R. G. E. (1980). Sites of metal deposition in the cell wall of *Bacillus subtilis*. *J. Bacteriol.* **141**, 876–887.

Beveridge, T. J., Meloche, I. D., Fyfe, W. S., and Murray, R. G. E. (1983). Diagenesis of metals chemically complexed to bacteria: Laboratory formation of metal phosphates, sulfides, and organic condensates in artificial sediments. *Appl. Environ. Microbiol.* **45**, 1094–1108.

Beveridge, T. J., and Murray, R. G. E. (1976). Uptake and retention of metals by cell walls of *Bacillus subtilis*. *J. Bacteriol.* **127**, 1502–1518.

Borrok, D., Fein, J. B., and Kulpa, C. F. (2004a). Proton and Cd sorption onto natural bacterial consortia: Testing universal adsorption behavior. *Geochim. Cosmochim. Acta* **68**, 3231–3238.

Borrok, D., Fein, J. B., and Kulpa, C. F. (2004b). Cd and proton adsorption onto bacterial consortia grown from industrial wastes and contaminated geologic settings. *Environ. Sci. Technol.* **38**, 5656–5664.

Borrok, D., Fein, J. B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M., and Kemner, K. (2004c). The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chem. Geol.* **209**, 107–119.

Borrok, D., Fein, J. B., and Turner, B. F. (2005). A universal surface complexation framework for modeling proton binding onto bacterial surfaces in geologic settings. *Am. J. Sci.* **305**, 826–853.

Boyanov, M. I., Kelly, S. D., Kemner, K. M., Bunker, B. A., Fein, J. B., and Fowle, D. A. (2003). Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray absorption fine structure spectroscopy study. *Geochim. Cosmochim. Acta* **67**, 3299–3311.

Brassard, P., Kramer, J. R., and Collins, P. V. (1990). Binding site analysis using linear programming. *Environ. Sci. Technol.* **24**, 195–201.

Camesano, T. A., and Logan, B. E. (2000). Probing bacterial electrostatic interactions using atomic force microscopy. *Environ. Sci. Technol.* **34**, 3354–3362.

Champ, D. R., Young, J. L., Robertson, D. E., and Abel, K. H. (1984). Chemical speciation of long-lived radionuclides in a shallow groundwater flow system. *Water Pollution Res. J. Can.* **19**, 35–54.

Champlin, J. B. F., and Eichholz, G. G. (1976). Fixation and remobilization of trace contaminants in simulated subsurface aquifers. *Health Phys.* **30**, 215–219.

Claessens, J., Behrends, T., and Van Cappellen, P. (2004). What do acid-base titrations of live bacteria tell us? A preliminary assessment. *Aquatic Sci.* **66**, 19–26.

Cox, J. S., Smith, D. S., Warren, L. A., and Ferris, F. G. (1999). Characterizing heterogeneous bacterial surface functional groups using discrete affinity spectra for proton binding. *Environ. Sci. Technol.* **33**, 4514–4521.

Daughney, C. J., and Fein, J. B. (1998). The effect of ionic strength on the adsorption of H$^+$, Cd^{2+}, Pb^{2+}, and Cu^{2+} by *Bacillus subtilis* and *Bacillus licheniformis*: A surface complexation model. *J. Colloid Interf. Sci.* **198**, 53–77.

Daughney, C. J., Fein, J. B., and Yee, N. (1998). A comparison of the thermodynamics of metal adsorption onto two gram-positive bacteria. *Chem. Geol.* **144**, 161–176.

Davis, J. A., Coston, J. A., Kent, D. B., and Fuller, C. C. (1998). Application of the surface complexation concept to complex mineral assemblages. *Environ. Sci. Technol.* **32**, 2820–2828.

Dittrich, M., and Sibler, S. (2005). Cell surface groups of two picocyanobacteria strains studied by zeta potential investigations, potentiometric titration, and infrared spectroscopy. *J. Colloid Interface Sci.* **286**, 487–495.

Ehrlich, H. L. (1996). "Geomicrobiology," 3rd ed. Marcel Dekker, New York.

Fein, J. B. (2000). Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chem. Geol.* **169**, 265–280.

Fein, J. B., Daughney, C. J., Yee, N., and Davis, T. (1997). A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochim. Cosmochim. Acta* **61**, 3319–3328.

Fein, J. B., Boily, J.-F., Yee, N., Gorman-Lewis, D., and Turner, B. F. (2005). Potentiometric titrations of *Bacillus subtilis* cells and a comparison of modelling approaches. *Geochim. Cosmochim. Acta* **69**, 1123–1132.

Fowle, D., and Fein, J. B. (1999). Competitive adsorption of metals onto bacterial surfaces: Application of a surface complexation approach. *Geochim. Cosmochim. Acta* **63**, 3059–3067.

Fowle, D., and Fein, J. B. (2000). Experimental measurements of the reversibility of metal-bacteria adsorption reactions. *Chem. Geol.* **168**, 27–36.

Francis, A. J., Gillow, J. B., Dodge, C. J., Harris, R., Beveridge, T. J., and Papenguth, H. W. (2004). Uranium association with halophilic and non-halophilic bacteria and archaea. *Radiochim. Acta* **92**, 481–488.

Freire-Nordi, C. S., Vieira, A. A. H., and Nascimento, O. R. (2005). The metal binding capacity of *Anabaena spiroides* extracellular polysaccharide: An EPR study. *Process Biochem.* **40**, 2215–2224.

Gannon, J. T., Manilal, V. B., and Alexander, M. (1991). Relationship between cell surface properties and transport of bacteria through soil. *Appl. Environ. Microbiol.* **57**, 190–193.

Gonçalves, M. L. S., Sigg, L., Reutlinger, M., and Stumm, W. (1987). Metal ion binding by biological surfaces: Voltammetric assessment in the presence of bacteria. *Sci. Tot. Environ.* **60**, 105–119.

Gorman-Lewis, D., Elias, P. E., and Fein, J. B. (2005). Uranium adsorption onto *Bacillus subtilis* bacterial cells. *Environ. Sci. Technol.* **39**, 4906–4912.

Gorman-Lewis, D., Fein, J. B., and Jensen, M. P. (2006). Enthalpies and entropies of proton and cadmium adsorption onto *Bacillus subtilis* bacterial cells from calorimetric measurements. *Geochim. Cosmochim. Acta* (in press).

Haas, J. R., Dichirstina, T. J., and Wade, R., Jr. (2001). Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chem. Geol.* **180**, 33–54.

Harden, V. P., and Harris, J. O. (1953). The isoelectric point of bacterial cells. *J. Bacteriol.* **65**, 198–202.

Harvey, R. W., George, L., Smith, R. L., and LeBlanc, D. R. (1989). Transport of microspheres and indigenous bacteria through a sandy aquifer: Results of natural- and forced-gradient tracer experiments. *Environ. Sci. Technol.* **23**, 51–56.

Harvey, R. W., Kinner, N. E., MacDonald, D., Metge, D. W., and Bunn, A. (1993). Role of physical heterogeneity in the interpretation of small-scale laboratory and field observations of bacteria, microbial-sized microsphere and bromide transport through aquifer sediments. *Water Resources Res.* **29**, 2713–2721.

Harvey, R. W., and Leckie, J. O. (1985). Sorption of lead onto two gram-negative marine bacteria in seawater. *Mar. Chem.* **15**, 333–344.

Hennig, C., Panak, P. J., Reich, T., Roβberg, A., Raff, J., Selenska-Pobell, S., Matz, W., Bucher, J. J., Bernhard, G., and Nitsche, H. (2001). EXAFS investigation of uranium(VI) complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochim. Acta* **89**, 625–631.

Huysmans, M., and Dassargues, A. (2005). Stochastic analysis of the effect of heterogeneity and fractures on radionuclide transport in a low-permeability clay layer. *Environ. Geol.* **48**, 920–930.

Jiang, W., Saxena, A., Song, B., Ward, B. B., Beveridge, T. J., and Myneni, S. C. B. (2004). Elucidation of functional groups on Gram-positive and Gram-negative bacterial surfaces using infrared spectroscopy. *Langmuir* **20**, 11433–11442.

Johnson, W. P., and Logan, B. E. (1996). Enhanced transport of bacteria in porous media by sediment-phase and aqueous-phase natural organic matter. *Water Res.* **30**, 923–931.

Kelly, S. D., Boyanov, M. I., Fein, J. B., Fowle, D. A., Yee, N., and Kemner, K. M. (2002). X-ray absorption fine structure determination of pH-dependent U-bacterial cell wall interactions. *Geochim. Cosmochim. Acta* **66**, 3855–3871.

Konhauser, K. O., Fyfe, W. S., Ferris, F. G., and Beveridge, T. J. (1993). Metal sorption and mineral precipitation by bacteria in two Amazonian river systems: Rio Solimoes and Rio Negro, Brazil. *Geology* **21**, 1103–1106.

Koretsky, C. (2000). The significance of surface complexation reactions in hydrologic systems: A geochemist's perspective. *J. Hydrol.* **230**, 127–171.

Kulczycki, E., Ferris, F. G., and Fortin, D. (2002). Impact of cell wall structure on the behavior of bacterial cells as sorbents of cadmium and lead. *Geomicrobiol. J.* **19**, 553–565.

Lau, T. C., Wu, X. A., Chua, H., Qian, P. Y., and Wong, P. K. (2005). Effect of exopolysaccharides on the adsorption of metal ions by *Pseudomonas sp* CU-1. *Water Sci. Technol.* **52**, 63–68.

Lower, S. K., Tadanier, C. J., and Hochella, M. F., Jr. (2000). Measuring interfacial and adhesion forces between bacteria and mineral surfaces with biological force microscopy. *Geochim. Cosmochim. Acta* **64**, 3133–3139.

Malard, F., Reygrobellet, J.-L., and Soulie, M. (1994). Transport and retention of fecal bacteria at sewage-polluted fractured rock sites. *J. Environ. Qual.* **23**, 1352–1363.

Markai, S., Andrès, Y., Montavon, G., and Grambow, B. (2003). Study of the interaction between europium (III) and *Bacillus subtilis*: Fixation sites, biosorption modeling and reversibility. *J. Colloid Interf. Sci.* **262**, 351–361.

Marques, A. M., Bonet, R., Simonpujol, M. D., Fuste, M. C., and Congregado, F. (1990). Removal of uranium by an exopolysaccharide from *Pseudomonas* sp. *Appl. Microbiol. Biotechnol.* **34**, 429–431.

Martinez, R. E., and Ferris, F. G. (2001). Chemical equilibrium modeling techniques for the analysis of high-resolution bacterial metal sorption data. *J. Colloid Interf. Sci.* **243**, 73–80.

Martinez, R. E., Smith, D. S., Kulczycki, E., and Ferris, F. G. (2002). Determination of intrinsic bacterial surface acidity constants using a Donnan shell model and a continuous pK_a distribution method. *J. Colloid Interf. Sci.* **253**, 130–139.

Matthess, G., and Pekdeger, A. (1985). Survival and transport of pathogenic bacteria and viruses in groundwater. *In* "Ground Water Quality" (C. H. Ward, W. Giger, and P. L. McCarty, Eds.), pp. 472–482. John Wiley and Sons, New York, NY.

McCarthy, J. F., and Zachara, J. M. (1989). Subsurface transport of contaminants. *Environ. Sci. Tech.* **23**, 496–502.

McDowell-Boyer, L. M., Hunt, J. R., and Sitar, N. (1986). Particle transport through porous media. *Water Resources Res.* **22**, 1901–1921.

Moysey, S., Caers, J., Knight, R., and Allen-King, R. M. (2003). Stochastic estimation of facies using ground penetrating radar data *Stochastic Environ. Res. Risk Assess.* **17**, 306–318.

Ngwenya, B. T., Sutherland, I. W., and Kennedy, L. (2003). Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Appl. Geochem.* **18**, 527–538.

Panak, P. J., Raff, J., Selenska-Pobell, S., Geipel, G., Bernhand, G., and Nitsche, H. (2000). Complex formation of U(VI) with *Bacillus*-isolates from a uranium mining waste pile. *Radiochim. Acta* **88**, 71–76.

Panak, P. J., Booth, C. H., Caulder, D. L., Bucher, J. J., Shuh, D. K., and Nitsche, H. (2002a). X-ray absorption fine structure spectroscopy of plutonium complexes with *Bacillus sphaericus*. *Radiochim. Acta* **90**, 315–321.

Panak, P. J., Knopp, R., Booth, C. H., and Nitsche, H. (2002b). Spectroscopic studies on the interaction of U(VI) with *Bacillus sphaericus*. *Radiochim. Acta* **90**, 779–783.

Payne, T. E., Davis, J. A., Ochs, M., Olin, M., and Tweed, C. J. (2004). Uranium adsorption on weathered schist: Intercomparison of modelling approaches. *Radiochim. Acta* **92**, 651–661.

Plette, A. C. C., van Riemsdijk, W. H., Benedetti, M. F., and Van der Wal, A. (1995). pH dependent charging behavior of isolated cell walls of a gram-positive soil bacterium. *J. Colloid. Int. Sci.* **173**, 354–363.

Plette, A. C. C., Benedetti, M. F., and van Riemsdijk, W. H. (1996). Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a gram-positive soil bacterium. *Environ. Sci. Technol.* **30**, 1902–1910.

Sarret, G., Manceau, A., Spadini, L., Roux, J.-C., Hazemann, J.-L., Soldo, Y., Eybert-Berard, L., and Menthonnex, J.-J. (1998). Structural determination of Zn and Pb binding sites in *Penicillium chrysogenum* cell walls by EXAFS spectroscopy. *Environ. Sci. Technol.* **32**, 1648–1655.

Small, T. D., Warren, L. A., Roden, E. E., and Ferris, F. G. (1999). Sorption of strontium by bacteria, Fe(III) oxide, and bacteria-Fe(III) oxide composites. *Environ. Sci. Technol.* **33**, 4465–4470.

Sokolov, I., Smith, D. S., Henderson, G. S., Gorby, Y. A., and Ferris, F. G. (2001). Cell surface electrochemical heterogeneity of the Fe(III)-reducing bacteria *Shewanella putrefaciens*. *Environ. Sci. Technol.* **35**, 341–347.

Songkasiri, W., Reed, D. T., and Rittmann, B. E. (2002). Bio-sorption of neptunium(V) by *Pseudomonas fluorescens*. *Radiochim. Acta* **90**, 785–789.

Stumm, W., and Morgan, J. J. (1996). "Aquatic Chemistry," 3rd ed. John Wiley & Sons, Inc., New York, USA.

Templeton, A. S., Spormann, A. M., and Brown, G. E. (2003). Speciation of Pb(II) sorbed by *Burkholderia cepacia*/goethite composites. *Environ. Sci. Technol.* **37**, 2166–2172.

Texier, A.-C., Andrès, Y., Illemassene, M., and Le Cloirec, P. (2000). Characterization of lanthanide ions binding sites in the cell wall of *Pseudomanas aeruginosa*. *Environ. Sci. Technol.* **34**, 610–615.

Toner, B., Manceau, A., Marcus, M. A., Millet, D. B., and Sposito, G. (2005). Zinc sorption by a bacterial biofilm. *Environ. Sci. Technol.* **39**, 8288–8294.

van der Wal, A., Norde, W., Zehnder, A. J. B., and Lyklema, J. (1997). Determination of the total charge in the cell walls of Gram-positive bacteria. *Colloids Surf. B Biointerfaces* **9**, 81–100.

Wasserman, E., and Felmy, A. R. (1998). Computation of the electrical double layer properties of semipermeable membranes in multicomponent electrolytes. *Appl. Environ. Microbiol.* **64**, 2295–2300.

Wasserman, E., Felmy, A. R., and Chilakapati, A. (2000). Non-equilibrium thermodynamic simulation of metal uptake in the bacterial electrical double-layer. *Colloids Surf. B Biointerfaces* **18**, 19–29.

Weppen, P., and Hornburg, A. (1995). Calorimetric studies on interactions of divalent cations and microorganisms or microbial envelopes. *Thermochim. Acta* **269/270**, 393–404.

Wightman, P. G., Fein, J. B., Wesolowski, D. J., Phelps, T. J., Benezeth, P., and Palmer, D. A. (2001). Temperature dependence of surface protonation reactions occurring at the bacteria-water interface. *Geochim. Cosmochim. Acta* **65**, 3657–3669.

Yee, N., Benning, L. G., Phoenix, V. R., and Ferris, F. G. (2004a). Characterization of metal-cyanobacteria sorption reactions: A combined macroscopic and infrared spectroscopic investigation. *Environ. Sci. Technol.* **38**, 775–782.

Yee, N., and Fein, J. B. (2001). Cd adsorption onto bacterial surfaces: A universal adsorption edge? *Geochim. Cosmochim. Acta* **65**, 2037–2042.

Yee, N., and Fein, J. B. (2003). Quantifying metal adsorption onto bacteria mixtures: A test and application of the surface complexation model. *Geomicrobiol. J.* **20**, 43–60.

Yee, N., Fowle, D. A., and Ferris, F. G. (2004b). A Donnan Model for metal sorption onto *Bacillus subtilis*. *Geochim. Cosmochim. Acta* **68**, 3657–3664.

Alfalfa Winter Hardiness: A Research Retrospective and Integrated Perspective[*]

Yves Castonguay,[1] Serge Laberge,[1]
E. Charles Brummer[2] and Jeffrey J. Volenec[3]

[1]Soils and Crops Research and Development Center,
Agriculture and Agri-Food Canada, Québec G1V 2J3, Canada
[2]Department of Agronomy, Iowa State University, Ames, Iowa 50011
[3]Department of Agronomy, Purdue University, West Lafayette, Indiana 47907

I. Introduction
II. Morphological and Developmental Bases of Winter Survival
    A. Crown Depth, Root Morphology, and Winter Survival
    B. Fall Dormancy and the Acquisition of Freezing Tolerance
    C. Impact of Environmental Factors on Alfalfa-Freezing Tolerance
III. Molecular Bases of Winter Survival: Current Understanding
and Emerging Concepts
    A. Tolerance to Freeze-Induced Desiccation and Cold Hardiness
       of Alfalfa
    B. Cold-Induced Accumulation of Cryoprotective Sugars
    C. Amino Acids
    D. Modification of Gene Expression at Low Temperature
IV. The Genetic Bases of Cold Adaptation in Alfalfa
    A. Genetic Variability for Freezing Tolerance
    B. Conventional Genetic Selection for Improved Winter Hardiness
       and Freezing Tolerance
    C. Marker-Assisted Selection
    D. Conceptual Approach to the Genetic Control of Freezing Tolerance
       in Alfalfa
    References

Insufficient cold hardiness is a major impediment to reliable alfalfa (*Medicago sativa* L.) production in northern regions experiencing harsh winter conditions. Numerous studies have documented the morphological and physiological traits associated with the acquisition of freezing tolerance and winter survival in alfalfa. Use of this information as selection criteria to breed cultivars

with superior winter hardiness has thus far been met with limited success. This can be attributed to many factors including: the large number of traits affecting winter survival; the multigenic nature of most traits, large environmental inter-actions, and an undesirable linkage between acquisition of freezing tolerance and fall growth cessation (fall dormancy). In the last two decades, the advent of molecular biology and quantitative genetic techniques has markedly increased our knowledge of the molecular and genetic bases of superior alfalfa winter hardiness. Our understanding of the mechanisms underlying the perception of the low temperature signal and its transduction into morphological and physi-ological responses leading to cold hardiness has progressed, but still remains fragmentary. Current evidence indicates that cold hardiness of alfalfa relies on tolerance to extensive freeze-induced desiccation. Low temperature-induced accumulation of soluble sugars and stress-related translation products were found to be, in some instances, more abundant in cold-tolerant cultivars and to be under some level of genetic control. Limited stability of these traits and conflicting reports on their relationship with freezing tolerance preclude their adoption as molecular screening tools. The development of robust screening techniques will require a more complete knowledge of the genetic bases of freezing tolerance. Heritability estimates suggest that independent selection for winter hardiness, freezing injury and autumn growth is possible, and that winter hardiness and autumn growth could be manipulated independently. This creates the opportunity to develop high-yielding cultivars with improved winter hardiness. A screening test for freezing tolerance performed under controlled conditions recently led to the development of populations with increased freez-ing tolerance and led to significant improvement in alfalfa winter survival. Unique genetic material, combined with novel gene discovery approaches, could be lead to the identification of genetic polymorphisms associated with freezing tolerance in alfalfa and pave the way to marker-assisted selection. Based on the current knowledge, we propose a conceptual framework for the genetic determination of cold adaptation of alfalfa.   © 2006, Elsevier Inc.

I. INTRODUCTION

Alfalfa is the most important forage legume grown in North America. It is grown on 9.5 million hectares in the United States (http://www.usda.gov/nass/), and 32 million hectares worldwide (Russelle, 2001). In recent years the cash value of alfalfa in the United States has been estimated at $8 billion, placing it third behind maize (*Zea mays* L.) and soybean (*Glycine max* L. Merrill). How-ever, this underestimates the actual value of alfalfa to US agriculture because most alfalfa production leaves the farm as meat and milk. Estimates of the value of forages to the ruminant livestock industry exceed $27 billion, which exceed the cash value of other crop commodities (Barnes and Nelson, 2003).

Alfalfa contributes to sustainable crop and livestock production systems in numerous ways. This includes N derived from biological fixation of atmospheric dinitrogen gas (N_2) by legume–*Rhizobium* symbioses, which

represents potentially a more sustainable approach to acquiring N than N derived from industrial sources (Crews and Peoples, 2004). As a result, perennial legume-based cropping systems have lower global warming potential than annual cropping systems (Robertson *et al.*, 2000). Historic trends for N inputs in farming systems indicate that N derived from biological N_2 fixation has declined from 50% in the 1950s to around 20% in the mid-1990s. In addition, forage production systems reduce soil erosion and protect surface and groundwater by capturing nitrate and water, and the large belowground biomass of alfalfa also sequesters vast quantities of C.

Regions that are most affected by insufficient alfalfa winter hardiness include the midwest United States, western and eastern Canada, and Northern Europe. Several severe winters in the 1990s resulted in significant stand losses in the midwest United States and Canada with as much as 1,000,000 ha killed in some years. With reestablishment costs at roughly \$400 ha^{-1} (Barnhart *et al.*, 2004), winterkill caused expenses approaching one-half billion dollars in these years. Modeling of global climate change suggests increased frequency and severity of alfalfa winter kill in future years because of less reliable autumn-hardening conditions coupled with reduced snow cover; both key factors in alfalfa winter hardiness (Bélanger *et al.*, 2002). As a result, understanding the morphological, physiological, and molecular mechanisms associated with alfalfa winter hardiness and freezing tolerance is key to long-term food security.

II.   MORPHOLOGICAL AND DEVELOPMENTAL BASES OF WINTER SURVIVAL

A.   CROWN DEPTH, ROOT MORPHOLOGY, AND WINTER SURVIVAL

Crown depth below the soil surface has historically been viewed as a key morphological adaptation for successful overwintering of forage legumes. The deeper-set crowns of fall-dormant alfalfa cultivars, when compared to the shallow-set crowns of nondormant cultivars, is thought to serve as an escape mechanism preventing exposure of overwintering tissues to sublethal temperatures (Smith, 1952). Despite its importance as an escape strategy for forage legumes, we do not understand the physiological and morphological mechanisms controlling contractile growth that ultimately results in crowns developing up to 2 cm below the soil surface.

The freezing and thawing of soil during winter can cause heaving during which crowns and the upper portion of the taproot are pushed vertically out of the soil, resulting in tissue exposure to ambient air temperatures with little or no insulating from the soil (Fig. 1). Early study described ice formation in soil and how it could result in heaving (Bouyoucos and McCool, 1928).
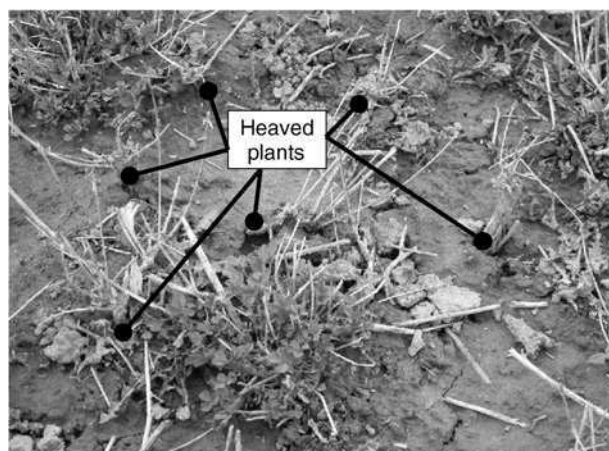
Figure 1 Heaving of soil exposed the crowns and upper portions of the taproots of alfalfa to lethal temperatures over winter resulting in a high frequency of plant death. Photo was taken in April in west-central Indiana, USA.

Needle-like ice crystals grow rapidly using water drawn to the surface via capillary action from deeper horizons in the soil profile. Capillary pores enlarge at their basal ends as additional ice is deposited, pushing the frozen soil surface and rooted plants upward. When the ice sheet melts, plants are left exposed above the soil surface. Because of greater temperature variation, more heaving generally occurs in sods with extensive areas of exposed soil that lack the insulating properties of dense sods (Decker and Ronningen, 1957). Because residue insulates the soil against temperature change, autumn cutting has been shown to be a major contributor to frost heaving of alfalfa (Belesky and Fedders, 1997; Van Keuren, 1988). In addition, autumn-harvested plants that heaved had less root mass and lower root total non-structural carbohydrate (TNC) concentrations. This is consistent with the early report of Willard (1930) that frequent cutting in summer (five times per year) in Ohio reduced root mass, root reserves, and enhanced winter killing caused by heaving. Residue also enhances snow cover that insulates the soil and crown against rapid changes in temperatures (Leep *et al.*, 2001). Winter survival of traditionally nonhardy alfalfa germplasms occurred in this Michigan study when at least 10 cm of snow was present.

Several factors are known to influence heaving of alfalfa plants. The taproot nature of alfalfa makes this species more susceptible to heaving, whereas branch-rooted legumes and fibrous-rooted grasses heave less (Johnson *et al.*, 1998; Kinbacher, 1956; Smith, 1951). Portz (1967) reported cultivar differences in frost heaving with fall-dormant cultivars heaving less than nondormant cultivars, which have less branch rooting in general.

Severing alfalfa taproots 14 cm below the soil surface caused extensive branching and reduced heaving when compared to plants with intact taproots (Klebesadel, 1964). Wiersma *et al.* (1997) reported cultivar differences in heaving and attributed 50% of the variation in heaving to resistance to Aphanomyces root rot caused by *Aphamomyces euteiches* Drechs. This disease reduces root branching resulting in susceptible plants having only a taproot. Perfect *et al.* (1987) also examined the relationship between taproot architecture and heaving. Alfalfa plants with taproots exhibited about 50% greater heaving than plants with the creeping root trait. Heaving-induced root injury varied with plant size; small plants had severed taproots while large plants had broken lateral roots. Root anchorage and tissue elasticity may be important factors influencing heaving/root injury. In contrast, Russell *et al.* (1978) reported no consistent difference in frost heaving among four alfalfa cultivars, including a rhizomatous variety.

Heaving is associated with poor soil drainage and diurnal temperatures that cycle from below to above freezing. The 30 winters in Illinois where heaving was observed on imperfectly drained claypan soils coincided with above-average rainfall from October to January and moderate winter temperatures. This close association between poor drainage and high incidence of heaving was later confirmed by Jones and Olsen (1987) who examined persistence over winter in southern Illinois of four semidormant cultivars on three soil types. Survival percentages over the first winter increased from 29 to 63–89% as drainage increased from poorly drained to moderately well drained to well drained. No cultivar differences in heaving resistance were observed. Deep tillage that mixed the A and B horizons of a silt loam soil has been shown to reduce heaving by improving water infiltration into the soil (Cary *et al.*, 1967). Clearly, multiple factors, including root architecture, soil drainage, and prevailing weather, interact in causing heaving of alfalfa.

B.   Fall Dormancy and the Acquisition of Freezing Tolerance

Fall dormancy more than any other morphological feature defines alfalfa adaptation to agroecosystems in northern latitudes. Early study by Timmons and Salmon (1932) reported that cultivars adapted to the southwest United States incurred greater injury and death than did dormant cultivars grown in the midwest United States. Fall-dormant cultivars exhibit reduced shoot elongation and decumbent shoot orientation in autumn and are very winter hardy (Sprague and Fuelleman, 1941). Fall nondormant cultivars have extensive shoot elongation in autumn, shoots with a vertical orientation, and generally poor survival over winter in northern regions (Fig. 2). Because shoot growth rate after cutting and flowering of nondormant cultivars
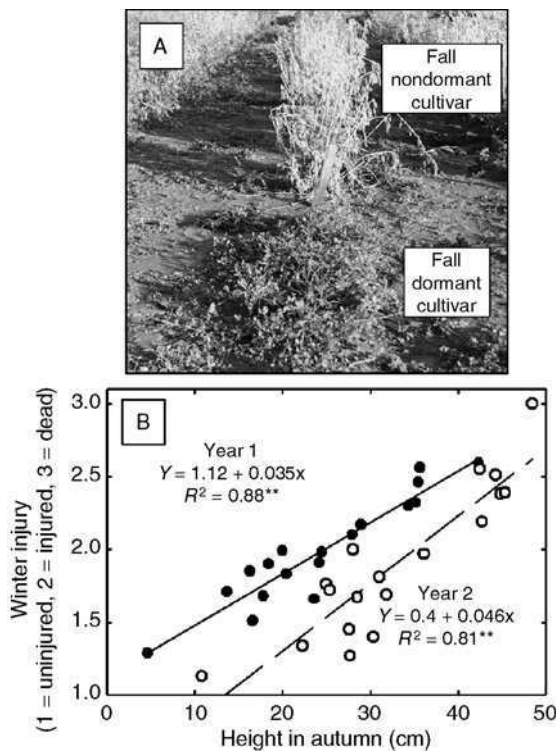
Figure 2 Large differences in the extent and orientation of shoot growth in autumn, known as "fall dormancy" also influences freezing tolerance with the tall, nondormant cultivar (A, rear) being more freezing sensitive than the fall-dormant cultivar (A, front). When diverse germplasms are evaluated for height-winterhardiness relationships (B), a linear increase in winter injury occurs as alfalfa shoot height increases. Adapted from Cunningham *et al.* (2001).

is faster in nondormant than dormant cultivars (Buller *et al.*, 1955), nondormant cultivars could result in higher forage yield, and historically there has been interest in using less fall-dormant cultivars in regions with significant winter stress (Marquez-Ortiz *et al.*, 1999). However, the risk of winter injury remains a significant deterrent to implementing this recommendation.

The negative association of nondormant growth in autumn and winter survival in alfalfa has been observed both for genotypes within a cultivar as well as among cultivars (Larson and Smith, 1963, 1964; Schwab *et al.*, 1996; Smith, 1964), suggesting that fall dormancy was a useful predictor of winter injury potential in regions with severe winters. Nevertheless, alfalfa genotypes with nondormant fall growth, which possess modest levels of winter hardiness, have been identified (Busbice and Wilsie, 1965; Daday, 1964).

Brummer *et al*. (2000) found little association between fall dormancy and winter survival in a segregating F_1 alfalfa population. This suggests that it may be possible to dissociate, to some extent, the genetic components of fall dormancy and winter hardiness, and simultaneously select for extensive autumn growth, good winter hardiness, and rapid shoot regrowth after harvest in summer (Brouwer *et al*., 2000). Weishaar *et al*. (2005) demonstrated that improved winter hardiness could be achieved through genetic selection within several fall nondormant alfalfa cultivars. Germplasms were identified that possessed better winter hardiness than would otherwise be predicted using fall dormancy. However, progress toward realizing this goal in commercial products has been slow with little deviation in the traditional fall dormancy-winter hardiness relationship being evident in released cultivars (Haagenson *et al*., 2003).

In spite of its importance in determining alfalfa adaptation to harsh winter conditions, the physiological basis for genetic differences in fall dormancy remains essentially unknown. Grafting has been used to understand root versus shoot effects on fall dormancy responses (Heichel and Henjum, 1990). Canopy morphology of grafted plants was primarily conditioned by the shoot genotype, with minor rootstock effects. Freezing tolerance of dormant crowns was reduced when grafted onto nondormant rootstocks, whereas freezing tolerance of nondormant crowns was not increased by grafting onto dormant rootstocks. Thus, both root and shoot effects condition fall dormancy responses and winter survival. Kanneganti *et al*. (1998a,b) modeled alfalfa-freezing injury, including the impact of fall dormancy. Their analysis revealed that dormant cultivars harden up to three times faster than nondormant cultivars. Hardening initiated at crown temperatures of 15°C, with maximum hardening rate occurring between 5 and 10°C and dehardening at temperatures greater than 15°C. They reported little increase in potential for winter injury as fall dormancy rating increased from 1 to 4 on a scale that range from 1 for highly dormant cultivars (0.5-cm regrowth 40 days after a cut in early September) to 9 (regrowth $\geq$ 40 cm) for the least dormant types (Teuber *et al*., 1998). However, a large increase in the potential for injury was observed as fall dormancy rating increases from 4 to >6. Freezing damage to cell membranes is often more important in fall nondormant cultivars when compared to fall-dormant cultivars (Larson and Smith, 1964). Fall dormant, winter-hardy cultivars have little postfreezing electrolyte leakage (an indirect indicator of injury), and low-protein levels and activity of malate dehydrogenase (MDH) in the root leachate (Sulc *et al*., 1991a,b).

Because of a general belief that taproot reserves play a key role in the determination of winter survival, initial spring herbage growth, and herbage regrowth after harvest in summer, the impact of fall dormancy on their accumulation has been evaluated in several studies. Larson and Smith

(1964) reported that taproot carbohydrate pools and macronutrient concentrations were not related to marked variations in winter injury between cultivars of contrasting fall dormancy ratings. However, concentrations of nitrogen (N) pools in November were higher in taproots of winter-hardy cultivars when compared to nondormant cultivars that were winter injured. Selection of germplasms with contrasting fall dormancy responses within various genetic backgrounds have been used to explore the relationship between fall dormancy and cold acclimation (Cunningham *et al.*, 1998). Selection for greater fall dormancy in CUF 101, a very fall nondormant cultivar, reduced shoot height in the autumn and increased winter survival from 1% to 95% (Fig. 3). Increase in fall dormancy within CUF 101, did not alter taproot starch concentrations, but increased soluble sugars and protein
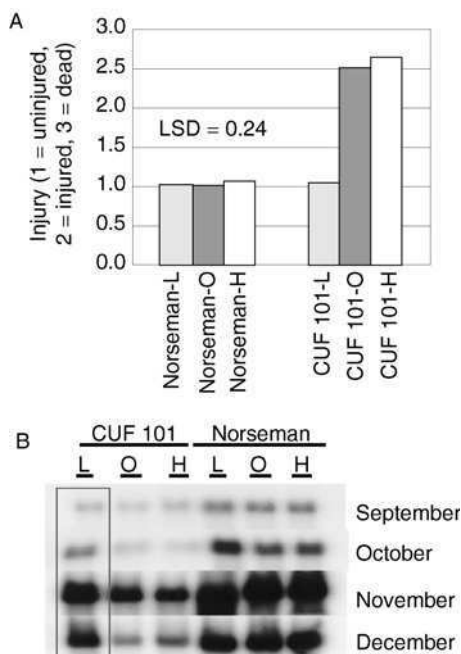
Figure 3 Influence of selection for fall dormancy on winter injury (A) and taproot transcript levels for *RootCAR1*, a gene whose expression is associated with genetic differences in winter hardiness. Plants from the original population (O) and selections from within that population for low (L) and high (H) fall growth were compared. While all Norseman germplasms are hardy and exhibit high *RootCAR1* transcript levels beginning in November, selection for greater fall dormancy (CUF 101-L) in the nondormant cultivar CUF 101 (CUF 101-O) reduced winter injury and enhanced *RootCAR1* transcript abundance in taproots (box). Selection for less fall dormancy in CUF 101 (CUF 101-H) did not alter height or *RootCAR1* transcript levels compared to CUF 101-O, both of which were low in December compared to CUF 101-L. Adapted from Cunningham *et al.*, 2001.

concentrations in taproots by 50% in December. Steady-state transcript level in December for a cold acclimation-responsive gene (*RootCAR1*) was also increased in taproots of CUF 101-derived populations selected for increased dormancy (Cunningham *et al.*, 2001). These observations agree with results obtained, using a wide range of alfalfa cultivars differing in fall dormancy (Haagenson *et al.*, 2003). Some of these physiological responses have also been observed during cold acclimation of suspension-cell cultures derived from fall dormant and fall nondormant alfalfa cultivars (Kalengamaliro *et al.*, 2000). Cells derived from a fall-dormant cultivar were more freezing tolerant than were cells derived from a fall nondormant cultivar, and these differences were positively associated with soluble sugar accumulation. However, patterns of protein accumulation and gene expression in cold-acclimating cells of dormant and nondormant plants did not agree with observations obtained using field-grown plants. This suggests caution be used when extrapolating findings from cell-culture studies to predict plant responses in the field.

C. Impact of Environmental Factors on Alfalfa-Freezing Tolerance

1. Photoperiod and Temperature and Acquisition of Freezing Tolerance

The environmental cues, leading to greater freezing tolerance of alfalfa, include both decreasing photoperiod and declining air temperatures. Photoperiod and temperature interact in a cultivar-specific manner resulting in enhanced winter hardiness of alfalfa. Rapid changes in photoperiod that occur at Northern latitudes, like Alaska, are not perceived properly by cultivars adapted to regions located in the continental United States, and these plants do not cold acclimate or survive winter even though other conditions (temperature) are appropriate (Bula *et al.*, 1956; Klebesadel, 1971). Thus, rate of photoperiod change appears to be more important than photoperiod per se in initiating cold acclimation. Reducing photoperiod without a reduction in temperature can increase alfalfa-freezing tolerance, but this response was cultivar specific (Hodgson, 1964). Cold resistance of fall nondormant cultivars, measured as electrolyte leakage from taproots, did not change with shortening photoperiods, whereas a significant decline in electrolyte leakage occurred for taproots of the winter hardy, fall-dormant cultivar ''Ranger.''

Temperature changes also have a key role in enhancing alfalfa cold-acclimation responses. Shih *et al.* (1965) indicated that cold temperatures are the primary cue that leads to the development and maintenance of alfalfa

cold hardiness. They observed a synergistic effect of cool temperatures and short photoperiods in reducing shoot height (an estimate of fall dormancy), and ultimately increasing winter hardiness. Differential accumulation of protein, RNA, and DNA was associated with genetic differences in cold acclimation and freezing tolerance. Thus, cold acclimation requires a significant expenditure of energy in overwintering organs. Swanson and Adams (1959) showed that temperature influenced taproot dark respiration rates more than photoperiod during cold hardening and that increased respiration was more pronounced in fall-dormant cultivars than in nondormant cultivars. Schonhorst *et al*. (1957) examined autumn shoot height of 10 alfalfa cultivars differing in fall dormancy to alterations in photoperiod and temperature. The largest cultivar-related differences in shoot height occurred for plants grown at 15.5°C under a 12-h photoperiod. These differences were accentuated if plants at 15.5°C were transferred to –1°C during the 12-h dark period. Similar increases in the intensity of cold-acclimation response have been observed with plants acclimated to temperatures just below freezing (Castonguay *et al*., 1995). Cold acclimation begins in autumn when the mean air temperature approaches 10°C and the acquisition of freezing tolerance accelerates as temperatures approach 5°C (McKenzie *et al*., 1988). Using electrical conductivity to relate changes in cold tolerance to acclimating temperatures, Woolley and Wilsie (1961) proposed cold unit accumulation as a model to predict the level of cold tolerance of alfalfa. They selected 15.6°C (60°F) at a 10-cm soil depth as the threshold temperature, and summed the ''cold units'' after September 1 in Ames, IA. Cold unit accumulation was closely associated with reduction in electrolyte leakage from taproots. Plants were considered completely cold acclimated after 100 cold units (in °F) had accumulated, at which time, autumn growth could be removed without risk of increased winter injury. This concept has not been tested thoroughly to determine its applicability to a broader array of agroecosystems, and as a result, this temperature-based cold-acclimation model remains location specific.

2.   Influence of Disease on Alfalfa-Freezing Tolerance

A wide range of biotic and abiotic factors can influence alfalfa-freezing tolerance and winter survival. The impact of disease on alfalfa winter survival is still poorly understood. The interactions among disease stress, winter hardiness, and stand age were recognized early in alfalfa cultivar development (Grandfield, 1934). Plant losses were attributed to three causes: interplant competition, bacterial wilt, and winter kill. Most plant death in Year 1 was due to interplant competition (90%), with no impact of disease, and a minor effect of winter kill (10%). As the stand aged, plant losses due to

winter kill increased, especially for plants that were susceptible to bacterial wilt. Although disease stress can be a factor in alfalfa winter hardiness, current approaches for breeding for high-disease resistance ratings have not generally improved alfalfa persistence; a component of which is winter survival (Volenec *et al.*, 2002). Regression analysis of persistence versus disease resistance rating revealed that only 24% of 37 trials gave the anticipated increase in stand density with an increase in disease resistance ratings. The remaining trials exhibited no relationship between both traits, and in 11% of the trials, stand persistence declined as disease resistance ratings increased. Clearly there is room to improve our understanding of how disease impacts alfalfa winter survival and long-term persistence.

3. Impact of Soil Moisture on Alfalfa-Freezing Tolerance

Poor drainage is known to reduce alfalfa persistence (Benoit *et al.*, 1967). As tile line spacing decreased from none to 61–31 m, winter kill concomitantly declined from 87% to 62–44%. Poor drainage results in water on the soil surface forming ice sheets that quickly result in alfalfa death in winter. Sprague and Graber (1943) reported that encasing alfalfa in ice for 20 days or more completely kill the plants. These authors determined that injury was caused by factors in addition to the accumulation of CO_2 and limitation of O_2, which required more than 20 days to be injurious. These results were confirmed by Smith (1952), where alfalfa encased in ice had 100% survival after 21 days, but 50% of plants died as duration of encasement was extended to 35 days. In this study, fall-dormant cultivars were found to be more tolerant of ice encasement than were nondormant cultivars. This differential survival might be attributable to higher rates of dark respiration allowing injurious levels of CO_2 to be reached sooner in nondormant than in fall-dormant cultivars. Ice sheet formation under saturated soil conditions for 50 days kill virtually all the alfalfa without impacting root moisture content (Freyman and Brink, 1967). Carbon dioxide levels in ice-covered soil increased to 5%, while O_2 concentrations were depleted to less than 10% in 50 days. Intermittent flushing of air, N_2, or O_2 through soil of ice-covered alfalfa (21 days) increased survival and plant vigor whereas intermittent flushing with CO_2 killed all plants. The authors concluded that ice sheets are barriers to CO_2 diffusion, permitting this gas to accumulate to levels that are toxic to alfalfa (Freyman, 1969). Using plants enduring simulated winter conditions in an unheated greenhouse, Bertrand *et al.* (2001, 2003) observed a significant reduction in regrowth of alfalfa progressively conditioned to declining O_2 and rising CO_2 during autumn hardening and subsequently maintained under anoxic conditions many weeks during winter. A switch to energy-inefficient anaerobic respiration accelerated the use of carbon and

nitrogen reserves with profound consequences with regard to substrate availability for regrowth and the accumulation of potentially phytotoxic end products like ethanol.

4. Fertilization and Alfalfa Winter Hardiness

Plant nutrition, and especially potassium (K) application, is one management factor that is generally thought to improve alfalfa winter hardiness. Wang *et al.* (1953) showed that raising pH from 5 to 6.5 reduced winter killing from 90% to 50%. Addition of P and K at pH 6.5 further reduced winter killing from 50 to less than 20%. These authors reported that low-taproot protein concentrations were associated with high-winter killing under low pH conditions. In contrast, most other reports indicate no impact, and occasionally a negative impact of enhanced P and K nutrition on alfalfa winter hardiness. For example, Megee (1935) reported that fertilization with 0-8-24 increased electrolyte leakage (an indirect indicator of injury) from taproots of cold-acclimated alfalfa when compared to unfertilized plants. As expected, the nondormant cultivar "Arizona Common" had much greater electrolyte leakage than the fall-dormant cultivar "Hardigan." Moisture content of taproots from dormant and nondormant cultivars did not differ but was less in both groups in winter than in early autumn and spring. Gross *et al.* (1958) studied eight alfalfa cultivars to determine how P and K influenced persistence and yield in Iowa. Plant density decreased with P fertilization and was not influenced by K fertilization. This agrees with a study in Alaska, where K increased forage yield but did not alter persistence (Klebesadel and Brinsmade, 1966). Interaction between cultivar and autumn-cutting management can influence the response of alfalfa to changes in soil fertility. While autumn-cutting management and P, K, and P + K fertility did not consistently influence alfalfa winter hardiness, plant density and forage yield of "DuPuits" alfalfa was reduced the following spring if forage was harvested mid-September in plots not fertilized with K (Tesar and Yager, 1985). Levels of root TNC were not altered by autumn-cutting management and K and P fertilization. These reports of minor effects of P and K on alfalfa winter hardiness agree with results from Indiana, where frequent plant counts revealed plant death occurring almost exclusively in summer and not in winter (Berg *et al.*, 2005). While K had no influence on alfalfa persistence in this study, plant densities were significantly reduced with P fertilizer applications. Nevertheless, forage yield was greatest for plots provided adequate levels of both P and K. Part of the confusion regarding the influence of P and K on alfalfa winter survival may result from inaccuracies associated with estimating plant densities by counting crowns from aboveground instead of directly digging and counting plants.

5. Autumn-Cutting Effects on Winter Hardiness and Taproot Reserves

Autumn-cutting strategies and their impact on alfalfa winter hardiness remains a topic of continued debate. Willard (1930) was among the first to propose that a period of uninterrupted growth in autumn was necessary for winter survival in Ohio so taproot reserves could accumulate prior to the onset of winter. Other work in Michigan showed that plants cut in September had fewer crown buds, fewer stems, and lower yield the next spring than plants left uncut or those cut in October (Silkett *et al.*, 1937). Electrolyte leakage from taproots and winter kill were greater for September-cut plants than uncut or October-cut plants. Not all findings indicate that autumn cutting leads to reduced winter survival and spring vigor. Plant persistence and taproot carbohydrate-reserve levels were not reduced by autumn cutting in Oklahoma (Sholar *et al.*, 1983) or Michigan (Tesar and Yager, 1985). Other studies indicate that autumn cutting, which follows intensive harvest schedules in summer (35 day or less), can reduce winter hardiness (Brink and Marten, 1989; Sheaffer *et al.*, 1992). Using cultivars that differ in fall dormancy Haagenson *et al.* (2003) reported that both winter hardiness and spring vigor in Indiana, United States are reduced by harvesting a fifth time (ca. 30-days intervals) in early October (Fig. 4).

One attribute of alfalfa that has long been the focus of studies on the mechanisms controlling alfalfa-freezing tolerance and winter hardiness is TNC reserves. Failure of alfalfa to survive winter has been generally attributed to low-TNC reserves in taproots at the onset of winter. Despite its wide acceptance by agronomists, there is little experimental evidence supporting this popular concept. Early work compared taproot TNC levels of legume species and found a positive relationship between TNC concentrations and species-related variation in winter hardiness (Bula and Smith, 1954). These authors also observed an extensive conversion of starch to sugars during cold acclimation, along with increased taproot N concentrations. Later work (Bula *et al.*, 1956) found no association between taproot TNC concentrations and winter survival among alfalfa cultivars. They, however, observed greater accumulation of N reserves in taproots of winter-hardy cultivars when compared to a nonhardy alfalfa cultivar adapted to the southwest United States. Mass of etiolated shoot growth from crowns of dark-grown alfalfa plants also has been used to assess the amount and availability of taproot organic reserves and its relationship to winter survival (Klebesadel, 1971). However, like TNC concentrations, the relationship between etiolated shoot growth from plants at winter dormancy and alfalfa-freezing tolerance is poor. While work confirms that root TNC concentration is not a good indicator of the genetic potential for freezing tolerance, other taproot constituents, including individual soluble sugars that comprise the
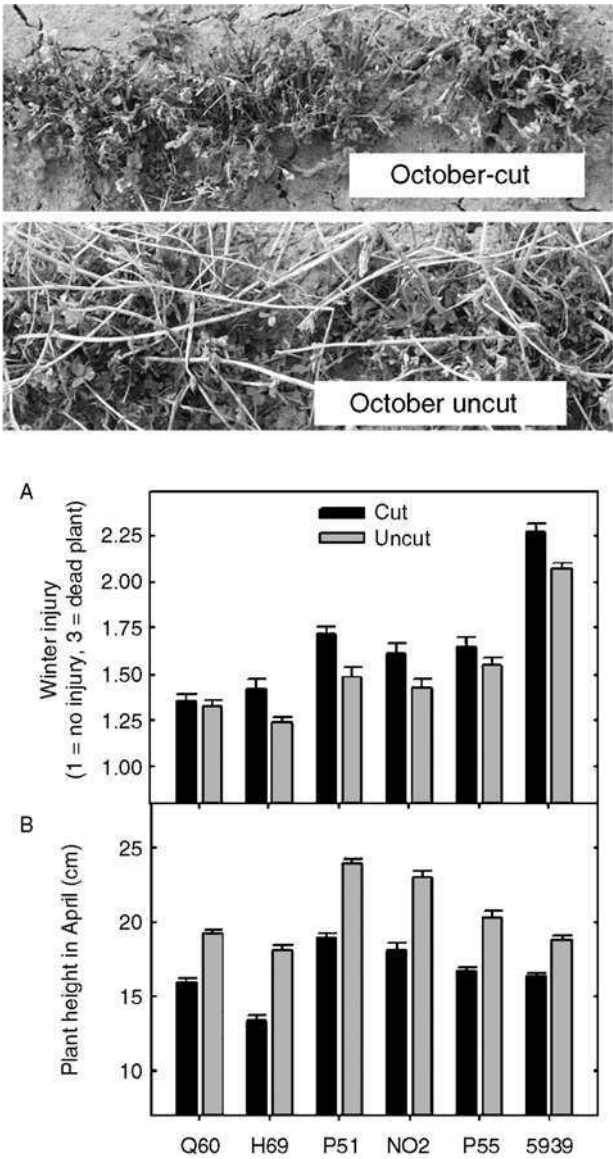
Figure 4 Influence of shoot removal in October on appearance and vigor of new shoots the following April (photo) and on winter injury and shoot height (a measure of spring vigor) in April. Cultivars differed in fall dormancy with Q60 being the most dormant (FD = 3) to 5939 (FD = 9). Adapted from Haagenson *et al.* (2003).

root TNC pool and several N pools, are better related to freezing tolerance (Castonguay *et al*., 1995; Cunningham *et al*., 1998, 2001; Dhont *et al*., 2003; Haagenson *et al*., 2003). Therefore, mechanistic models describing alfalfa winter hardiness and freezing tolerance must include factors beyond the traditional notion that root TNC reserves control alfalfa persistence.

III. MOLECULAR BASES OF WINTER SURVIVAL: CURRENT UNDERSTANDING AND EMERGING CONCEPTS

A. TOLERANCE TO FREEZE-INDUCED DESICCATION AND COLD HARDINESS OF ALFALFA

Freezing of water is the single most critical event in respect to cold hardiness of plants (Junttila and Robberecht, 1999). McKenzie *et al*. (1988) pointed out that winterhardiness of alfalfa is determined to a large extent by its freezing tolerance and the improvement of this trait should result in superior field survival. The paradigm on the physiological and biochemical traits required for freezing temperature tolerance is based on the recognition that (1) the plasma membrane is the primary site of freezing injury and (2) maintenance of the semipermeability of cell membranes after a freeze–thaw cycle is a prerequisite to plant survival (Steponkus and Webb, 1992). Detailed aspects of cell water relations in frozen tissues have been extensively reviewed by Guy (1990), Reaney and Gusta (1999), and Mazur (2004). Intracellular freezing being a lethal event, cold-adapted plants have evolved mechanisms to allow either substantial supercooling of the cell solution or to initiate ice nucleation in the extracellular spaces. The exact causes of freezing damage in many herbaceous perennial plants, including alfalfa, are still undetermined. In supercooled tissues, heterogeneous nucleation in the presence of ice nucleators can trigger lethal intracellular freezing. However, it is assumed that damage largely occurs as a result of extracellular freezing that imposes a dehydrative force on the unfrozen solution and ultimately kills cells (Pearce, 2004). In some cases, damage can be caused by the growth of extracellular ice masses that crush tissues. The freeze-induced desiccation of the cytosol is driven by the lower chemical potential of ice than that of water at a given temperature. The vapor pressure deficit that is thus generated between the extracellular spaces and liquid water inside the cell rapidly expands with decreasing temperatures generating extensive desiccation forces. Pearce (1988) estimated that at a temperature of $-18.6\,^{\circ}C$, which cold-acclimated rye (*Secale cereale* L.) and alfalfa can tolerate for short period of time, about 90% of cell water has been lost with a concomitant

large reduction in cell volume. The hypothesis that tolerance to freezing and desiccation share, to some extent, the same molecular bases of adaptation is supported by observations that limited desiccation can increase freezing tolerance at temperatures that normally would not induce cold acclimation (Cloutier and Andrews, 1984). Exogenous application of the water stress-related growth regulator abscisic acid (ABA) can induce freezing tolerance in whole plants (Rikin et al., 1975) and cell cultures (Orr et al., 1985) of alfalfa. Cold-inducible proteins and genes were also shown to be synthesized in ABA-treated cells of alfalfa in the absence of low-temperature induction (Mohapatra et al., 1988a,b). However, Mohapatra et al. (1988a) noted only a partial increase in freezing tolerance in ABA-treated cells highlighting the specificity of the low-temperature signal and its requirement for the development of maximum freezing tolerance.

Theories that relate the manifestation of injuries to the dehydration of the plasma membrane or that emphasize the damaging effects of the concentration of intracellular solutes in the unfrozen cell solution are still debated (Mazur, 2004). Ultimately, cold-induced desiccation will affect the functional properties of macromolecules and will promote phase separation or the formation of nonlamellar domains in membranes (Uemura et al., 1995). Consequently, key adaptive traits for superior cold tolerance of alfalfa are expected to be found among factors controlling the location, the growth rate, and the propagation of ice within the plants and among molecular changes that will help stabilize membranes and proteins in the dehydrated state. Thus, freezing tolerance is a complex trait that requires a coordinated set of physiological and biochemical mechanisms and the regulation of numerous genes.

B. COLD-INDUCED ACCUMULATION OF CRYOPROTECTIVE SUGARS

Low temperature-induced accumulation of soluble sugars plays an important role in the acquisition of cold tolerance in plants (Guy, 1990). One of the most common and abundant soluble sugars that accumulates at low temperature is sucrose (Suc). The protective action of cold-induced accumulation of soluble sugars could derive from their colligative effect, reducing the amount of water lost during extracellular freezing (Santarius and Giersch, 1983). Noncolligative mechanisms whereby soluble sugars could help stabilize macromolecules and membranes in freeze-desiccated cells have also been proposed (Anchordoguy et al., 1987; Crowe et al., 1992). These two mechanisms might occur simultaneously and results from the action of different types of soluble sugars (Pearce, 1999).

Starch hydrolysis with the concomitant accumulation of large amounts of nonreducing soluble sugars, mostly Suc, are known to occur in crowns and

taproots of alfalfa and have been positively associated with winterhardiness (Castonguay *et al.*, 1995; Cunningham *et al.*, 1998, 2001; McKenzie *et al.*, 1988). Cunningham *et al.* (2001) reported a strong negative relationship ($r^2 = 0.92$) between taproot sugar concentration and winter injury. Winter-hardy cultivars and germplasms had taproot sugar concentrations in excess of 100 mg g^{-1} dry weight, whereas plants that exhibited severe injury and death over winter had taproot sugar concentrations of 50 mg g^{-1} dry weight or less. Early onset of taproot sugar accumulation was also positively associated with genetic variation in winter hardiness (Cunningham *et al.*, 1998). Fall-dormant, winter-hardy cultivars have a nearly linear increase in taproot sugar concentrations between late summer and late autumn, whereas nondormant, alfalfa cultivars begin to accumulate sugar in taproots during the last month of autumn. The maximum accumulation of soluble sugars in crowns of field-hardened alfalfa coincided with the occurrence of maximum freezing tolerance in winter (Paquin and Lechasseur, 1982). Sucrose accumulation during cold acclimation of alfalfa has been linked to a rise in sucrose phosphate synthase (SPS) activity, a key enzyme in Suc biosynthesis (Castonguay and Nadeau, 1998). Increases in freezing tolerance correlating with leaf Suc content have been observed in transgenic *Arabidopsis* overexpressing SPS (Strand *et al.*, 2003). The SPS activity in *Deschampia antartica*, a very freezing-tolerant grass with an unusually high content of Suc in leaves could not be related to an increase in SPS gene expression or protein content highlighting the importance of nontranscriptional controls of this enzyme activity (Zúñiga-Feest *et al.*, 2005). Although Suc typically accumulates to very high concentrations in cold-hardened alfalfa, Castonguay *et al.* (1995) reported a lack of relationship between midwinter freezing tolerance and Suc levels in crowns (Fig. 5A). This observation clearly indicates that variation in the level of this disaccharide is not a determinant factor of superior winter hardiness of alfalfa. Analysis of the relationship between freezing tolerance and elevated Suc in transgenic *Arabidopsis* with suppressed vacuolar invertase activity suggested an indirect contribution of Suc to the cold-acclimation process (Klotke *et al.*, 2004). These authors proposed that the adaptive value of cold-induced Suc accumulation could be partly mediated through its impact on the synthesis of other key metabolites like raffinose, proline (Pro), and glutamine.

Increase in raffinose family oligosaccharides (RFO), which include the sucrosyl-galactosides raffinose and stachyose, have been related to the acquisition of tolerance to a number of environmental stresses, including dehydration (Downie *et al.*, 2003), heat (Panikulangara *et al.*, 2004), and cold (Liu *et al.*, 1998; Stushnoff *et al.*, 1998). Castonguay *et al.* (1995) observed a close relationship between the capacity of alfalfa cultivars to accumulate small amounts of the oligosaccharides stachyose and raffinose and freezing tolerance (Fig. 5B). These RFO initially undetectable in
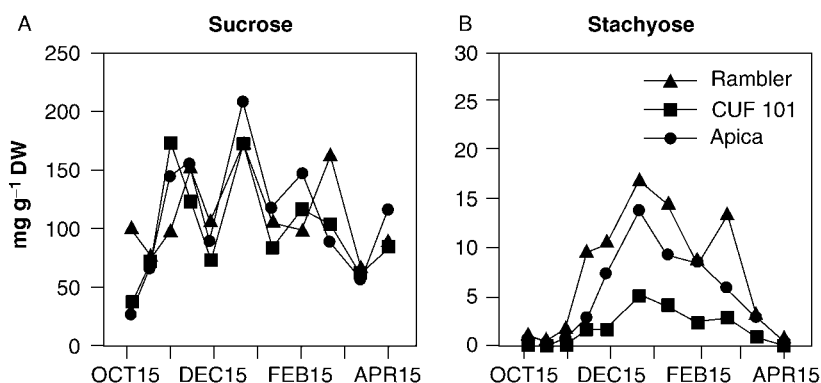
Figure 5 Accumulation of Suc and stachyose (mg g^{-1} dry weight) in crowns of very hardy (Rambler), hardy (Apica), and nonhardy (CUF 101) alfalfa cultivars during cold acclimation in autumn and the reduction in sugar levels as plants deharden in spring. Adapted from Castonguay and Nadeau (1998).

unacclimated crowns of alfalfa, accumulate later in autumn than Suc at the time when soil temperatures are near freezing. Cunningham *et al.* (2003) reported a positive association between RFO accumulation in December and genetic differences in winter survival of alfalfa providing further evidence for an adaptive role in the determination of winter hardiness potential (Fig. 6). It has been postulated that RFO confer stress protection through the promotion of a glassy state that helps protect macromolecular structures in desiccated cells (Leopold, 1990). Alternatively, they may help stabilize membranes by directly interacting with polar head groups of phospholipids (Crowe *et al.*, 1992).

The synthesis of raffinose, the first member of the RFO series, results from the transfer of a galactosyl unit from the donor molecule galactinol to Suc (Peterbauer and Richter, 2001). Larger RFO are produced by the sequential transfer of additional galactose from the same donor molecule or through a galactinol-independent pathway involving the transfer of galactose unit from one RFO molecule to another (Bachman *et al.*, 1994). Galactinol is synthesized from UDP-galactose and *myo*-inositol by the action of galactinol synthase (GaS). GaS catalyzes the first committed step in the synthesis of RFO and is thought to play a key regulatory role in that pathway (Peterbauer and Richter, 2001). A central role for GaS is confirmed by the numerous reports of a positive correlation between GaS activity and RFO content (Castonguay and Nadeau, 1998; Handley *et al.*, 1983; Saravitz *et al.*, 1987). The induction of the RFO synthesis pathway during cold acclimation has been associated with an increase in the levels of the GaS transcripts in *Arabidopsis* (Klotke *et al.*, 2004; Liu *et al.*, 1998)
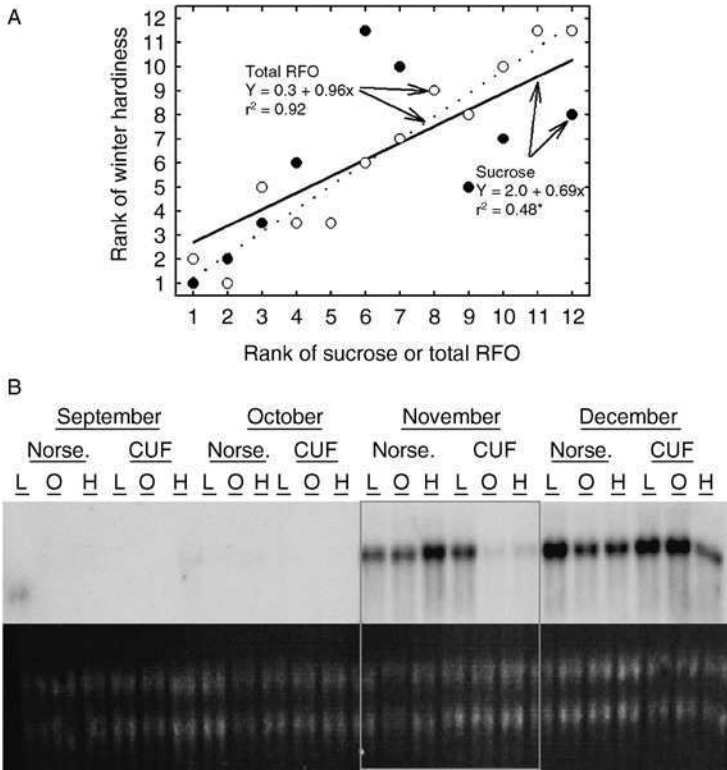
Figure 6 Relationship between rank of winter hardiness and Suc or raffinose family oligo-saccharide (RFO, sum of raffinose and stachyose) concentrations in taproots of alfalfa germplasms selected for contrasting fall dormancy (A). Transcript levels for galactinol synthase (B), the first committed step in RFO synthesis, are higher in November for the fall dormant, winter hardy-Norseman and the fall dormant, winter-hardy selection of CUF 101 (CUF 101-L, see Fig. 3) when compared to the nondormant germplasms CUF 101-O and CUF 101-H (box). Adapted from Cunningham *et al.* (2003).

and alfalfa (Cunningham *et al.*, 2003) (Fig. 6). A close link between RFO accumulation and stress tolerance is further supported by an enhanced tolerance to drought in transgenic plants of *Arabidopsis* overexpressing a drought-induced GaS (Taji *et al.*, 2002).

Warm temperature regulates RFO catabolism by increasing the activity of α-galactosidase (α-gal). This enzyme hydrolyzes RFO during deacclimation releasing the terminal galactose moieties. Pennycooke *et al.* (2004) observed a rapid increase in α-gal transcripts in cold-acclimated plants of petunia (*Petunia x hybrida* "Mitchell") within 1 h after transfer to warm temperature. The induction of α-gal expression was accompanied by an

increase in the enzymatic activity and a decrease in raffinose content. Down-regulation of α-gal in transgenic petunia led to an increase in freezing tolerance, whereas its overexpression caused a decrease in both raffinose levels and freezing tolerance (Pennycooke et al., 2003). Activity of α-gal increased sharply during spring dehardening of alfalfa and was coincident with the disappearance of RFO (Castonguay and Nadeau, 1998). However, the fact that α-gal activity did not differ between three alfalfa cultivars with contrasting RFO accumulation during winter indicates that this enzyme is not a major metabolic control point of the genetic variability for their accumulation.

In spite of the numerous reports on the adaptive value of cold-induced accumulation of soluble sugars, and more specifically RFOs, uncertainties still remain on their overall contribution to the determination of cold tolerance in alfalfa. For instance, Haagenson et al. (2003) observed an unexpected relationship between increased taproot soluble sugars (Suc and RFO) and reduced winter survival in alfalfa that had been defoliated in autumn. This was consistent with the separate report by Dhont et al. (2002) of increases in alfalfa root sugar levels in two winter-hardy cultivars that were defoliated in autumn. This apparent contradiction might be partly resolved by the observation by Dhont et al. (2006) that winter survival, as indicated by plant density in the spring, was closely related to higher concentrations of soluble sugars in roots of alfalfa that had been defoliated early in the previous autumn. This could also reflect the fact that freezing tolerance involves multiple mechanisms, of which nonreducing sugars constitute only one factor. In addition, selection for greater winter hardiness does not always result in accumulation of sugars, proteins, and amino acids in all genetic backgrounds (Weishaar et al., 2005). This implies that multiple, but equally effective, cold-acclimation responses may exist in this species. Nevertheless, based on the cumulated evidence on their adaptive value, there should be focused efforts to identify and characterize allelic forms of GaS and α-gal genes associated with superior RFO levels in alfalfa. Once they are characterized, these genes could then be used to alter RFO metabolism and help clarify their cold-adaptive value and the eventual benefits of this trait in the improvement of freezing tolerance of alfalfa.

C.   AMINO ACIDS

Free amino acids were shown to accumulate in taproots and crowns during cold acclimation of alfalfa (Dhont et al., 2003; Hendershot and Volenec, 1993). A marked increase in free Pro in roots of alfalfa during cold acclimation has long been documented (McKenzie et al., 1988; Paquin, 1984). Proline is a widespread compatible osmolyte that accumulates to high

concentrations in many plant species following exposure to environmental stress (Rai, 2002). Whether Pro accumulation confers stress tolerance or is a consequence of stress-induced metabolic imbalance is still a matter of debate (Lutts et al., 1999; Wanner and Junttila, 1999). Many functions have been assigned to Pro, such as mediator of osmotic adjustment (Handa et al., 1986), a free radical scavenger (Hong et al., 2000), a macromolecular structure stabilizer (Ain-Lhout et al., 2001), a redox potential buffer (Hare et al., 1998), and a nitrogen source in stressed cells (Chiang and Dandekar, 1995). Direct evidence supporting the role of Pro in stress tolerance was provided by mutant and transgenic Pro-overproducing plants (Dörffling et al., 1993; Hong et al., 2000; Roosens et al., 2002; Summaryati et al., 1992).

In higher plants, Pro is synthesized by either the glutamate or ornithine pathway. The enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS), which catalyzes a rate-limiting step in Pro synthesis in the glutamate pathway, is critical to plant response to abiotic stresses (Hong et al., 2000; Kavi Kishor et al., 1995). Activity of P5CS was shown to be cold inducible and essential for low temperature ($4°C$) tolerance of rice (Oryza sativa L.) (Hur et al., 2004). Conflicting observation that genetic variability in osmotically induced Pro accumulation in rice cultivars of contrasting cold tolerance was not related to a change in P5CS regulation raised questions on the general adaptive value of this enzyme (Hien et al., 2003). Two P5CS genes are present in the Medicago sativa and Medicago truncatula genomes, and Pro accumulation under osmotic stress was preceded by an increase in P5CS transcript level in both species (Armengaud et al., 2004; Ginzberg et al., 1998). Proline also can be synthesized from ornithine through the action of the enzyme ornithine δ-amino transferase (δ-OAT). Both ornithine and glutamate pathways were shown to be regulated at the transcriptional level in response to osmotic stress, and to contribute to Pro accumulation in M. truncatula (Armengaud et al., 2004). Transgenic lines of Arabidopsis, expressing an antisense construct of the Pro-degrading enzyme Pro dehydrogenase, were found to be more tolerant to freezing and high salinity than wild-type plants (Nanjo et al., 1999). No information is available on the transcriptional regulation of Pro synthesis or degradation in Medicago sp. at low temperature.

Apart from Pro, the concentrations of arginine (Arg) and histidine (His) also markedly increased in alfalfa taproots during cold acclimation in autumn (Dhont et al., 2003). Although the kinetics of Arg accumulation was consistent with a potential association with the acquisition of cold tolerance, further investigation will be required to elucidate its function in overwintering plants. Svenning et al. (1997) noted superior Arg accumulations in winter-hardy populations of white clover (Trifolium repens L.). A clearer understanding of the protective role(s) of cold-induced accumulation of Pro, Arg, and His and the mechanisms that control their

accumulation are prerequisites to their use as selection criteria in plant-breeding programs or their overproduction in transgenic plants as strategies to improve winterhardiness of alfalfa.

D.   MODIFICATION OF GENE EXPRESSION AT LOW TEMPERATURE

With the advent of molecular biology, significant insights have been gained in the last 25 years in our understanding of the genetic bases of the cold-acclimation process in plants (Thomashow, 2001). The hypothesis that the acquisition of cold tolerance during hardening in autumn relies on the transcriptional activation of a set of genes with the ensuing accumulation of new proteins was initially proposed by Weiser (1970). The first experimental evidence to support Weiser's hypothesis of altered gene expression in plants exposed to low temperature was the report by Guy *et al*. (1985) on the accumulation of new translatable mRNAs in spinach leaves exposed to low temperature. Numerous studies since then have confirmed the occurrence of cold-induced accumulation of new transcripts in many plant species, and extend the concept of low temperature induced changes in gene expression also to include the downregulation of transcription of a specific set of genes (Pearce, 2004). Although transcriptional regulation has often been shown to be involved, changes in gene expression also may result from alterations in the stability and translatability of mRNA at low temperature (Gilmour *et al*., 1988). A great diversity of cold-regulated (COR) genes have been isolated and characterized, and in some cases relevant information has been gained on the function of the protein products (Thomashow, 1998). The products of these genes can be broadly categorized into proteins that mechanistically protect plants against environmental stresses or that control the expression of target genes (Seki *et al*., 2004). However, very little is still known on the adaptive value of COR genes and their relative contribution to the development of freezing tolerance. Consequently, practical applications using this large body of knowledge are still lacking, although promising approaches are emerging. Research strategies are attempting to distinguish changes in gene expression that alter growth at low temperature or resistance to low-temperature pathogens from those that have a major impact on the development of freezing tolerance. As pointed out by Xin and Browse (2000), the assessment of the relative contribution of each COR gene is a formidable challenge often met with limited success since multiple genes are expected to act in concert to increase freezing tolerance.

A significant amount of research has long been devoted to the elucidation of the genetic bases of cold acclimation in alfalfa. The analysis of soluble proteins composition during cold hardening of alfalfa revealed qualitative and quantitative variations in profiles of protein extracts from cold-hardened

and control plants (Coleman *et al.*, 1966; Gerloff *et al.*, 1967). Many quantitative and qualitative differences between cold-tolerant and cold-sensitive alfalfa cultivars were revealed through the analysis of hydrolytic enzymes profiles (Krasnuk *et al.*, 1978). The observation of an additional esterase band uniquely detected in a cold-tolerant cultivar was the first instance of a potential link between the accumulation of a specific gene product and cold-hardiness potential in alfalfa. Using *in vivo* and *in vitro* labeling of polypeptides, Mohapatra *et al.* (1987a,b) obtained confirmatory evidence that several proteins were increasingly or newly synthesized during cold acclimation of alfalfa seedlings and related some of these changes to the induction of a new set of translatable mRNAs. Castonguay *et al.* (1993, 1997b) confirmed the occurrence of extensive changes in the populations of translatable mRNAs extracted from crowns of alfalfa cold-acclimated under either environmentally controlled or natural winter conditions. These studies identified a number of COR translation products that were more abundant or that specifically accumulated in cold-tolerant cultivars.

Freezing-tolerant and freezing-sensitive cultivars of alfalfa appear to share similar COR gene complements; however, noticeable differences exist in the rate and extent of expression in response to cold. Castonguay *et al.* (1998) documented extensive genotypic variability for cold-induced accumulation of COR gene products and RFO within populations of alfalfa. Marked differences in the cold-induced accumulation of RFO between propagated clones from genotypes selected for their contrasting RFO levels confirmed that variations in molecular changes that occur at low temperature are under some level of genetic control. In other words, genes present in the genome of cold-sensitive cultivars or genotypes within cultivars fail to be expressed in a timely manner in response to environmental cues. Kaur and Gupta (2005) point out that although most of the biochemical mechanisms required for the acquisition of stress tolerance are present in all species, the ability to perceive and transduce the external signals into a series of molecular events leading to physiological responses can differ widely among genotypes within a species. An understanding of the genetic determinants of these differences could have important implications in plant-breeding programs and the development of biotechnological approaches for the improvement of winter hardiness.

1. Low-Temperature Signal Transduction

In spite of the major research efforts being devoted to the study of the control of gene expression, signal-transduction pathways involved in the activation of COR genes are still largely unknown. Advances are slowed by the complexity and multilevel redundancy of the controls. Under a given

set of environmental conditions, the expression of genes involved in different physiological processes or that accumulate in a specific cell type or plant organ must be coordinated by different signal mechanisms. There is a growing body of evidence that the mechanisms controlling gene expression vary considerably between genes (Hirt, 1999). Different stresses provide cells with different information making stress-signaling pathways a very complex and intricate web (Knight and Knight, 2001). There also are indications for the occurrence of significant overlap between drought, salinity, cold, and ABA signal-transduction pathways (Kreps *et al.*, 2002). In that context, it is difficult to sort out key regulatory genes that control the expression of genes of interest among the whole cascade of events.

Increased cytosolic calcium (Ca^{2+}) concentration, as a key component of the signal-transduction pathway for low temperature and other environmental stresses, has been well documented (Sanders *et al.*, 1999). The low-temperature signal is initially perceived through its effect on membrane fluidity with increased rigidity promoting acclimation and enhanced fluidity preventing it (Sangwan *et al.*, 2002). Increased membrane rigidity can trigger a rapid and transient increase in cytosolic Ca^{2+} through an influx from the apoplast and evoke changes in membrane potential that may act as a primary stress signal (Krol *et al.*, 2004). These early events are followed by the production of secondary messengers and growth regulators like inositol triphosphate (IP_3) and ABA that stimulate further influx of Ca^{2+}. Ultimately, the Ca^{2+} signal is transduced via a cascade of protein phosphorylation involving calcium sensors (e.g., calmodulin, calcineurin B-like proteins), protein kinases, and protein phosphatases that can activate transcription factors and induce changes in gene expression (Kaur and Gupta, 2005).

Research conducted with alfalfa cell-suspension cultures has contributed significantly to the elucidation of the essential role of Ca^{2+} in the cold-acclimation process. For instance, the use of the Ca^{2+} channel blocker lanthanum (La^{3+}) to prevent its influx from the apoplast or antagonists of calmodulin and Ca^{2+}-dependent protein kinases (CDPK) concomitantly inhibited the development of freezing tolerance, cold-induced changes in protein phosphorylation, and the accumulation of transcripts of COR genes (Monroy *et al.*, 1993a). Conversely, artificially induced increases in cytosolic Ca^{2+} in the absence of cold temperatures triggered the expression of COR genes (Monroy and Dhindsa, 1995). A reorganization of the cytoskeleton was shown to be an important component of low-temperature transduction in alfalfa cell-suspension cultures, establishing a link between increased membrane rigidity and Ca^{2+} influx at low temperature (Örvar *et al.*, 2000). Strong upregulation of a CDPK at low temperature (Monroy and Dhindsa, 1995) and cold-inactivation of protein phosphatase 2A (Monroy *et al.*, 1998) provides additional support to a role for Ca^{2+}-induced changes in protein phosphorylation as an important component of the cold-acclimation

response of alfalfa. A sequence of low-temperature signaling events involving COR protein phosphorylation in alfalfa was further substantiated by the transient cold-activation of a specific mitogen-activated protein kinase (MAPK) (Jonak *et al*., 1996). The MAPKs are an important part of signal transduction networks and have been ubiquitously involved in plant responses to environmental stresses (Hirt, 2000). The low-temperature induction of the alfalfa MAPK has been linked to increased membrane rigidity using chemical modulators of membrane fluidity (Sangwan *et al*., 2002).

The transcriptional control of stress-regulated genes typically requires the binding of transcription factors to target *cis*-acting elements located upstream of their coding sequence in the promoter region. Transcription factors modulate gene expression through sequence-specific DNA binding and/or protein–protein interactions (Zhang *et al*., 2005). A number of transcription factors that belong to various gene families have been shown to activate the expression of stress-regulated genes (Kizis *et al*., 2001). The C-repeat–binding factor (CBF)/dehydration response element binding (DREB) that belong to the APETALA 2 (AP2)/ethylene response element-binding factor (EREBP) family of proteins is by far the best understood and most extensively studied stress-regulated transcriptional system in plants. The CBF genes were initially shown to be rapidly and transiently induced by cold and to activate the expression of a number of target genes in *Arabidopsis* (Thomashow, 2001). The expression of more than 100 genes known as the CBF regulon is induced by CBF-transcription factors (Maryama *et al*., 2004; Vogel *et al*., 2005). Low-temperature expression of CBF is itself under the regulation of positive (Chinnusamy *et al*., 2003; Zarka *et al*., 2003) and negative (Novillo *et al*., 2004; Vogel *et al*., 2005) transcriptional controls. Ectopic expression of CBF/DREB genes in plants improves cold tolerance in the absence of low-temperature stimulus (Jaglo-Ottosen *et al*., 1998). This increase in freezing tolerance is associated with the production of cryoprotective polypeptides and the accumulation of compatible solutes like Suc, raffinose, and Pro (Gilmour *et al*., 2000, 2004). These observations are consistent with the induction of GaS transcription (Gilmour *et al*., 2004) and extensive metabolome reconfiguration (Cook *et al*., 2004) mediated by CBF expression in nonacclimated plants of transgenic *Arabidopsis*. Components of the CBF/DREB cold-response pathway are entirely or partly conserved in many plant species, including the cold-tolerant *Brassica napus*, wheat (*Triticum aestivum* L.), rye (Jaglo *et al*., 2001), and barley (*Hordeum vulgare* L.) (Choi *et al*., 2002) and the chilling-sensitive maize (*Z. mays* L.) (Qin *et al*., 2004b), rice (Choi *et al*., 2002), tomato (*Lycopersicon esculentum* Mill.) (Zhang *et al*., 2004b), and pepper (*Capsicum annuum* L.) (Yi *et al*., 2004). This indicates that the CBF-response pathway cannot by itself confer freezing tolerance and that the presence of adaptive genes that are responsive to transcriptional regulators is required for the development

of cold tolerance. CBF-homologues have been found in EST collections of both *M. truncatula* (http://www.comparative-legumes.org/) and *M. sativa* (S. Laberge, personal communication). Zhang *et al.* (2005) reported that the overexpression of a putative AP2 domain-containing transcription factor gene isolated from the model legume *M. truncatula* enhanced drought tolerance in transgenic alfalfa. Whether the CBF-response pathway is operative during cold acclimation of alfalfa still remains to be established.

Although considerable attention is being devoted to the CBF/DREB family of transcription factors, there is evidence that multiple CBF-independent pathways are also involved in the cold-acclimation response (Kreps *et al.*, 2002; Vogel *et al.*, 2005; Zhu *et al.*, 2004). Fowler and Thomashow (2002) noted that many COR genes do not contain the C-repeat (CRT) element recognized by CBF in their promoter regions and that the CBF regulon highlighted by microarray analysis of CBF transgenic plants does not include all COR genes. Based on bioinformatic analysis of 514 COR transcripts, Vogel *et al.* (2005) concluded that *Arabidopsis* COR genes could be classified into seven distinct expression groups, and identified multiple potential *cis*-acting cold-regulatory elements. This estimate is supported by evidence for the involvement of other families of transcription factors in the mediation of cold acclimation, including homeodomain transcription factors (Zhu *et al.*, 2004), zinc-finger proteins (Kim *et al.*, 2001, 2005; Mukhopaday *et al.*, 2004; Sakamoto *et al.*, 2004; Vogel *et al.*, 2005), MYB transcription factors (Mattana *et al.*, 2005; Zhu *et al.*, 2005b), NAC domain protein family (Nogueira *et al.*, 2005), and basic helix-loop-helix proteins (Wang *et al.*, 2003). A MYB transcription factor was shown to regulate the expression of low-temperature genes through the control of ABA biosynthesis and acquisition of tolerance to freeze-induced dehydration. Many COR genes were shown to be inducible by ABA as well as by cold (Shinozaki and Yamaguchi-Shinozaki, 2000) and allelic *Arabidopsis* mutants defective for a key regulator of ABA biosynthesis also exhibit impaired expression of genes induced by cold and osmotic stress (Xiong *et al.*, 2001). A soybean (*G. max* L. Merrill) zinc-finger protein, SCOF-1, interacts with a transcription factor that bind to ABA-responsive element (ABRE) and enhances its binding to ABRE (Kim *et al.*, 2001). Constitutive expression of the *SCOF-1* gene improved cold tolerance in transgenic plants of *Arabidopsis* in the absence of low-temperature stimulus.

Whether the inheritance of specific forms of kinases or phosphatases, or activity of transcription factors is related to genetic variability of the cold-acclimation response within and among alfalfa populations/cultivars is still unknown. This certainly constitutes a logical target for research, considering the potential implications of identifying upstream regulators of the cold-acclimation process in the development of genetic material with superior freezing tolerance. The observation by Kawczynski and Dhindsa (1996) of cold stimulation of protein phosphorylation that was associated with

enhanced accumulation of cold-inducible, boiling-stable proteins in the freezing-tolerant alfalfa cultivar Apica to a greater extent than in the cold-sensitive cultivar Trek revealed the existence of clear genetic differences in cold signal response. Selections by Cunningham *et al.* (1998) for contrasted fall dormancy within parental lines having large initial differences in cold-acclimation responses constitute unique genetic resources to probe the molecular and genetic bases of alfalfa response to environmental changes in autumn and the subsequent acquisition of freezing tolerance.

2.   Identification and Functions of COR Genes

Twenty years of research on the identification and characterization of COR genes in plants have yielded a huge amount of information that still needs to be understood in order to establish their potential usefulness of these genes in molecular breeding. Initially, information was obtained on a few COR genes at a time by the differential screening of complementary deoxyribonucleic acids (cDNAs) libraries and the sequencing of a small number of purified DNA inserts. The pace of discovery rapidly accelerated in the mid-1990s with the advent of high-throughput sequencing of randomly selected cDNA clones referred to as expressed sequence tags (ESTs) and the capacity to simultaneously hybridize thousands of DNA sequences on high-density grids to obtain an assessment of global changes in the transcriptome (Richmond and Sommerville, 2000).

Comparative analysis of ESTs, using *Arabidopsis* gene arrays, have been used to achieve genome-scale probing of the changes in gene expression in plants exposed to environmental stresses (Fowler and Thomashow, 2002; Seki *et al.*, 2001, 2002). This molecular approach was successfully applied for the discovery of genes involved in the cold-acclimation process and provided significant insight into the complexity of this trait (Seki *et al.*, 2004). Simultaneous analysis of changes in expression of thousands of *Arabidopsis* genes spotted on a cDNA microarray with transcripts extracted from plants acclimated at $4^{\circ}C$ revealed that $\approx 4\%$ were cold-responsive with up to 70% of these genes being upregulated and 30% being downregulated (Fowler and Thomashow, 2002; Vogel *et al.*, 2005). Many of these genes have been assigned putative roles on the basis of their homology with other sequences reported in GenBank and were sometimes shown to be part of pathways leading to the modification in the composition of major classes of metabolites or to be involved in the transduction of the low-temperature signals (Seki *et al.*, 2004). Many of these cold-induced changes in transcript accumulation that occur in *Arabidopsis* are also observed during cold acclimation of other species (Dhanaraj *et al.*, 2004). Nevertheless, our understanding of the genetic control of cold tolerance and the adaptive value of COR genes remains fragmentary. Even in the case where their putative role fits with the

understanding of the physiological and molecular bases of cold adaptation, the *in vivo* function(s) of COR genes have seldom been confirmed (Volenec *et al*., 2002). Comparisons of global gene profiling between experimental treatments and contrasted genetic backgrounds will help identify candidate genes associated with variations in cold tolerance.

Even though transcript profiling is a powerful tool for identifying candidate genes, it has a number of inherent limitations with regard to the identification of adaptive genes (Richmond and Somerville, 2000). The technique provides a steady-state assessment of RNA levels and cannot always capture dynamic changes in the populations of translatable mRNAs in response to developmental and environmental signals. In addition, key information on changes in gene expression occurring in specific tissue or cells is often lost because entire plants or organs are used in transcript analyses. These limitations, combined with cross-hybridization between gene family members or specific allelic forms, can hinder identification of unique DNA sequences. Nevertheless, gene expression profiling constitutes a very sensitive tool to study phenotypic plasticity and it provides insight into genes responsible for variation of adaptive traits (Howe and Brunner, 2005). New oligonucleotide-based approaches allowing the specific representation of highly homologous DNA sequences on microarrays will help solve some of the cross-hybridization problems in the future (Seki *et al*., 2004). Sequence analyses of COR cDNAs from alfalfa revealed that many of these genes encode putative polypeptides homologous to stress- and developmentally regulated proteins (Castonguay *et al*., 1997a).

  a.   Dehydrins.   COR DNA sequences-encoding proteins with dehydrin-like motifs have been isolated from cold-acclimated alfalfa and were shown to be markedly upregulated in cold-acclimated plants (Haagenson *et al*., 2003; Wolfraim and Dhindsa, 1993; Wolfraim *et al*., 1993). Dehydrins, also known as Group 2 (D-11 family) late embryogenesis abundant (LEA) proteins, are stress-responsive proteins induced in response to dehydration, low temperature, exogenous ABA application, and wounding signals (Wise, 2003). The LEA proteins have been ubiquitously found in a wide range of organisms that undergo a dehydration-based stress, including herbaceous and woody plants, algae, prokaryotes, and animals (Bartels and Sunkar, 2005; Battista, 2001; Browne *et al*., 2002; Close, 1996). They are largely hydrophilic peptides with an abundance of charged and polar amino acid residues that allow them to remain stable under denaturing conditions (Close, 1996). In the absence of an apparent catalytic function, a number of protective mechanisms have been proposed, including water replacement, stabilization of membranes and macromolecules, ion sequestration as well as antifreeze, and radical-scavenging activities (Bartels and Sunkar, 2005; Hara *et al*., 2004; Wisniewski *et al*., 1999).

Dehydrin genes cosegregated with chilling tolerance in cowpeas [*Vigna unguiculata* (L.) Wasp.] (Ismail *et al.*, 1999), and their expression was related to the acquisition of drought tolerance among seven wheat cultivars (Lopez *et al.*, 2003). A close association between the levels of a 25-kDa dehydrin and the degree of leaf-freezing tolerance was observed in a segregating F2 population generated from a cross between a very hardy and a hardy *Rhododendron* species (Lim *et al.*, 1999). This relationship was later confirmed in a comparative assessment of the accumulation of the 25-kDa dehydrin in nonacclimated versus cold-acclimated leaf tissues of six *Rhododendron* species possessing contrasted freezing tolerance (Marian *et al.*, 2004). Overexpression of several groups of LEA proteins in transgenic plants, including the dehydrins, have provided compelling evidence for a protective role for these proteins to a number of environmental stresses, including water deficit (Park *et al.*, 2005), salt (Park *et al.*, 2003), and cold (Hara *et al.*, 2003; Puhakianen *et al.*, 2004). Constitutive expression of a wheat dehydrin gene in strawberry (*Fragaria x ananassa* Duch.) resulted in a 5°C improvement of freezing tolerance of cold-acclimated leaves (Houde *et al.*, 2004). No difference in freezing tolerance was found between wild-type and transgenic plants under nonacclimating conditions suggesting that additional cold-inducible factors are required for the protective action of the wheat dehydrin. Several lines of evidence indicate that LEA proteins may act synergistically in combination with other factors, including nonreducing sugars to protect extensively desiccated cells (Browne *et al.*, 2002; Oliver *et al.*, 2001; Wolkers *et al.*, 2001). The central role of dehydrins is further supported by preliminary observations from global gene expression studies with cold-acclimated alfalfa showing that dehydrins are the most highly expressed genes in cold-acclimated alfalfa (Laberge, personal communication).

b.   Antioxidant Defenses.   Increased levels of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radicals and hydroxyl radicals, are typically observed in plants under abiotic stress and may interfere with redox homeostasis of cells. The ROS can cause unspecific oxidation of key cell constituents like DNA, proteins, and membrane lipids (McKersie and Leshem, 1994). To cope with oxidative stresses plants have evolved a number of nonenzymatic (e.g., ascorbate, glutathione, polyamines, polyphenols, and so on) and enzymatic defense mechanisms. Thus, it is not surprising that proteins regulating the intracellular level of ROS, such as catalase, ascorbate, and glutathione peroxidases, and superoxide dismutase (SOD), are being found in cDNA libraries from plants exposed to abiotic stresses (Wong *et al.*, 2005). Transgenic plants with suppressed H_2O_2-scavenging capacity were more sensitive to abiotic stresses (Willekens *et al.*, 1997). Conversely, overexpression of H_2O_2-scavenging enzymes led to increase tolerance to abiotic stresses (Yan *et al.*, 2003). McKersie *et al.* (1993)

reported superior regrowth after freezing in transgenic alfalfa constitutively expressing a Mn-SOD from *Nicotiana plumbaginifolia*. Field assessment of alfalfa transformants indicated that SOD overexpression improved, in some cases, the vigor and field survival of the plants but that this response was not related to changes in freezing tolerance (McKersie *et al.*, 1996, 1999).

Polyamines have been found to accumulate under both biotic and abiotic stresses and to confer protection against many environmental stresses (Capell *et al.*, 2004; Kasinathan and Wingler, 2004). Although their protective mechanism is still unclear, it has been suggested that they might act as ROS scavengers (Drolet *et al.*, 1996; Ye *et al.*, 1997). Nadeau *et al.* (1987) documented an increase in the levels of free polyamines, mainly putrescine in cold-acclimated alfalfa. In a follow-up study performed under field conditions, Nadeau and Paquin (1988) confirmed the increase in putrescine in cold-hardened alfalfa at the end of winter and its rapid decline during spring dehardening.

   c.   **Antifreeze Proteins.**   Many plant species that survive the formation of extracellular ice secrete antifreeze proteins (AFPs), also called thermal hysteresis proteins, into their apoplast. The AFPs can bind onto the surface of ice and inhibit crystal growth *in vitro* (Griffith *et al.*, 2005). The accumulation of AFPs was correlated with freezing tolerance in winter and spring varieties of rye, wheat, and barley (Antikainen and Griffith, 1997). Bravo and Griffith (2005) have reported that *D. antarctica*, one of the only two vascular plants found in the Maritime Antarctica constitutively accumulates AFPs as part of its freezing-tolerance mechanisms. However, no antifreeze activity was detected in *Colobanthus quitensis*, the other Antarctic species. That result was not unexpected considering that antifreeze activity has thus far been detected in only about half of the overwintering plants in temperate regions with many dicotyledonous plants lacking antifreeze activity (Doucet *et al.*, 2000). Antifreeze activity has been assessed in leaf, root, and stem tissues of alfalfa collected during winter in the United Kingdom using an ice recrystallization assay (Doucet *et al.*, 2000). The observed lack of antifreeze activity in alfalfa might have been attributable to the mild hardening conditions to which these samples were exposed. Castonguay *et al.* (1993) have shown that superior freezing tolerance and associated changes in gene expression occur in plants of alfalfa acclimated at subfreezing temperatures. Confirmation of lack of antifreeze activity and AFP in alfalfa will require further *in vitro* assays since putative AFP homology cannot be assigned from database searches (Griffith and Yaish, 2004).

   d.   **Pathogenesis-Related Proteins.**   Cold acclimation increases not only tolerance to freezing, but also promotes nonspecific resistance to low-

temperature pathogens (Griffith and Yaish, 2004). A number of pathogenesis-related (PR) proteins, including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, and key enzymes, involved in the production of phyto-alexins are upregulated in response to both biotic and abiotic stresses (Yun et al., 1997). Studies provide evidence for interaction between biotic and abiotic stress-signaling pathways (Guo et al., 2004a; Narusaka et al., 2004; Yi et al., 2004). In addition to their role in pathogen resistance, additional functions, including antifreeze activity (Yeh et al., 2000) and cryoprotection (Hincha et al., 2001), have been documented for PR proteins. Meuriot et al. (2004) reported typical vegetative storage protein (VSP) behavior for a Type III chitinase that accumulates in taproots of alfalfa in autumn.

The cold inducible *msaCID* gene from alfalfa (Castonguay et al., 1997a) is homologous to the PR-10 family of small (15–18 kDa) host-encoded intracellular polypeptides (Linthorst, 1991). Members of this gene family are cold-induced in a number of species. A PR-10 homologue that accumulates in the cortical parenchyma cells of mulberry (*Morus bombycis* Koidz.) tree during winter also exhibited *in vivo* cryoprotective activity (Ukaji et al., 2004). Another cold-inducible gene from alfalfa, *msaCIA* (Laberge et al., 1993) that encodes a glycine-rich protein of unknown function was found to be homologous to glycine-rich peptides from the roots of *Capsella bursa-pastoris* that have strong antibacterial activity against gram-negative bacteria and fungi (Park et al., 2000). Dhont et al. (2006) showed that the expression of the *msaCIA* gene in winter-hardened alfalfa could be markedly reduced by defoliation in autumn. Couture et al. (2002) reported an increase in the severity of *Fusarium* root rot and crown rot diseases in autumn-defoliated alfalfa. Whether autumn defoliation reduces the ability of plants of alfalfa to withstand pathogen infection during winter because of reduced levels of PR proteins is certainly an area that deserves further investigation.

e. Genes of Unknown Function. No homology or assigned function has thus far been established for a large proportion of COR genes (Pearce, 2004). In blueberry (*Vaccinium* sp.), 43% of the high-quality sequences obtained from a cDNA library derived from cold-acclimated tissues did not have significant homology to sequences in GenBank (Dhanaraj et al., 2004). The functional assessment of these genes and understanding of their impact on stress tolerance presents an enormous challenge to scientists trying to unravel the molecular bases of superior cold hardiness (Zhu and Provart, 2003). This daunting task will benefit from an access to well-characterized phenotypes and mutants, and will necessitate the concerted research efforts of agronomists, physiologists, molecular biologists, and geneticists working in close collaboration.

A number of genes with no clear functions have been isolated from cold-acclimated alfalfa (reviewed in Castonguay *et al.*, 1997a). For instance, the *cas15/msaCIB/CAR1* gene code for a glycine-rich protein putatively targeted to the nucleus (Cunningham *et al.*, 2001; Monroy *et al.*, 1993b). Ferrullo *et al.* (1997) confirmed that the encoded MSACIB protein accumulates during cold acclimation of alfalfa and that although it is likely involved in the acquisition of freezing tolerance, much remains to be done to elucidate its cellular function(s).

f. The Adaptive Value of Cold-Induced Changes in Gene Expression. Mohapatra *et al.* (1989) published the first report on the molecular cloning of cold acclimation-specific genes that were isolated from the winter hardy *Medicago falcata* cv Anik. In that study a positive relationship was established between the levels of expression of three COR genes and variations in freezing tolerance among four alfalfa cultivars (Anik, Iroquois, Algonquin, and Trek). The observation of high-correlation co-efficients between the expressions of COR sequences and freezing tolerance led the authors to suggest their potential use as probes in breeding for increased freezing tolerance. Monroy *et al.* (1993b) also noted parallel changes in transcript levels of cas15, a cold-induced gene of unknown function, and variations of freezing tolerance in the winter-hardy cultivar Apica (Michaud *et al.*, 1983). However, the relative amount of cas15 transcripts did not vary among three cultivars with contrasting levels of freezing tolerance (Mohapatra *et al.*, 1989). A positive association between *RootCAR1*, a homologue of cas15, and alfalfa winter survival was subsequently observed among populations selected for contrasting fall dormancy (Cunningham *et al.*, 2001). Contradictory observations on the relationship between COR gene expression and cold hardiness of alfalfa is likely related to the fact that the genetic material compared in these studies not only differed with regard to their freezing tolerance potential but also differed in other attributes, including onset and extent of fall dormancy.

IV.   THE GENETIC BASES OF COLD ADAPTATION IN ALFALFA

A.   Genetic Variability for Freezing Tolerance

Alfalfa originated in the general area of the Caucasus Mountains, eastern Turkey, and northern Iran, and from there radiated throughout Eurasia, into the Arabian Peninsula, and across North Africa (Michaud *et al.*, 1988). Thus, alfalfa germplasm useful for modern agriculture, derives from plant

material naturally adapted to a broad range of conditions, from the dry subtropics to the tundra in northern Eurasia. As a consequence of the long period of natural selection that occurred in this germplasm, a relationship between growth habit, biomass production, autumn dormancy, and winter hardiness was developed. Particular germplasm from northern Siberia, when grown in the midwestern United States, begins to slow aboveground growth in early August, nearly a month before adapted cultivars show autumn induced changes, while germplasm from the Arabian Peninsula continues to grow vigorously until killed by frost.

1.   Test Winters and Field Selection

In general, selection for winter hardiness is done by evaluating breeding nurseries in the field for several years; plants surviving after this period of time are assumed to have sufficient winter hardiness for the particular zone of adaptation in which they are growing. Implicit in this methodology, however, is that the winters that the plants experience will be representative of the winters a released cultivar will face in farmers' fields. Ideally, plants will face a truly harsh winter—a test winter—in which they will be exposed to the full range of winter hardiness-related stresses, from freezing temperatures, severe desiccation, freeze–thaw cycles, early spring frost, ice sheeting, limited insulating snow cover, and possibly others. A test winter would be one that would kill cultivars with low-winter hardiness and provide a clear separation in survival percentages among cultivars differing in winter hardiness (McKenzie *et al.*, 1988). Because these conditions are extremely variable year-to-year, and because different combinations and severities of these stresses occur each year, field evaluations for multiple years are typically deemed necessary in order to select for improved winter hardiness with assurance.

2.   Freezing Tests Under Controlled Conditions

Screening tests performed under controlled conditions would enable more specific germplasm screening without relying on the occurrence of a test winter. Because freezing tolerance is perhaps the core trait associated with winter hardiness, most effort has focused on developing screening procedures for it.

Measuring the electrical conductivity of a solution containing electrolytes leaked from frozen roots is the most commonly used method to assess alfalfa-freezing tolerance in the laboratory (Brouwer *et al.*, 1998, 2000;

Bula *et al.*, 1956; Dexter *et al.*, 1932; Duke and Doehlert, 1981; Krasnuk *et al.*, 1978; Sulc *et al.*, 1991a,b). This test involves freezing roots at –8°C for 2–20 h, incubating the roots in distilled water at 4°C for 2–20 h, and measuring the conductivity of the resulting leachate at 25°C. The congruent result of all experiments is that more freezing tolerant (i.e., more winter hardy) germplasm has a lower specific conductivity than less hardy germplasm because fewer electrolytes (e.g., K^+) have leaked from the cells. Although leakage could be due to either membrane disruption or reversible solute movement, Sulc *et al.* (1991b) strongly implicated the former with their observation that MDH activity increased in the leakage. Although –8°C provides consistent results, maximum differentiation among cultivars may be achieved at temperatures lower than –8°C for hardy germplasm and higher than –8°C for nonhardy plant material (Sulc *et al.*, 1991b).

Other laboratory tests have been used to assess winter hardiness. Most importantly, absorbance of light at 265 nm of the leaked substances produced through the normal freezing test described in earlier section was highly correlated with electrical conductivity, suggesting it may be a more rapid and simple method of assessing freezing tolerance (Sulc *et al.*, 1991a). Similarly, the MDH activity in leakage could also be used as a measure of freezing tolerance, but the assay is more complex than either conductance or absorbance, and hence, is of less practical utility (Sulc *et al.*, 1991a). Finally, the germination of seeds that had been placed in an increasing osmotic gradient produced with sodium chloride and Suc was reduced less in winter hardy than in nonwinter-hardy cultivars (Rodger *et al.*, 1957). While this test appears simple and rapid, it has not been widely used in subsequent research.

B. CONVENTIONAL GENETIC SELECTION FOR IMPROVED WINTER HARDINESS AND FREEZING TOLERANCE

1. Quantitative Inheritance of Winter Hardiness

Winter hardiness, freezing injury, and autumn growth are all complex traits under quantitative genetic control. Although heritability estimates are difficult to compare due to differences in plot types, number of testing locations, and experimental designs, all traits have been shown to have moderate heritability, suggesting that selection would be effective in improving each of them individually. In an F_1 population derived from two semidormant genotypes, the broad sense heritability, which describes whether the trait is under genetic control, for winter injury was 0.73 based on entry means from five-clone plots grown in four replications at each of two

locations and 0.39 on the basis of a single five clone plot (Brummer et al., 2000). These results suggest that the evaluation of single plants, as is commonly done in alfalfa breeding, is unlikely to provide a clear picture of their winter hardiness, particularly after only 1 year. In the same study, entry mean heritability for autumn regrowth was 0.69 and plot heritability was 0.29. Other estimates of heritabilities based on entry means include 0.78–0.80 for cold injury (Daday, 1964), 0.65 for winter injury (Brouwer et al., 2000), 0.45 and 0.53 for autumn growth (Brouwer et al., 2000), 0.16 and 0.19 for freezing injury (based on electrical conductivity) (Brouwer et al., 2000), 1.32 for specific conductivity, and 0.72 for autumn height (Perry et al., 1987). Narrow-sense heritabilities, those based on variance of breeding values rather than total genetic variance, were only computed in the latter case, using parent-offspring regression.

Autumn regrowth appears to be primarily under additive genetic control and general combining ability (GCA) effects are present for autumn growth (Daday and Greenham, 1960; Perry et al., 1987; Riday and Brummer, 2002b; Theurer and Elling, 1963). However, specific combining ability effects (SCA) were present in some experiments, although they were of lesser importance than GCA effects. The presence of some nonadditive genetic control of autumn regrowth is also supported by our observation of negative heterosis for autumn regrowth in crosses between M. sativa subsp. sativa and M. sativa subsp. falcata (Riday and Brummer, 2002b).

Winter injury, frost tolerance, and/or freezing injury appear to be under partial to complete dominance. Heterosis for winter hardiness, whereby the F_1 hybrid progeny suffer less winter injury than either parent, has been noted in intersubspecies crosses (Riday and Brummer, 2002b), similar to that found in Arabidopsis hybrids between the Columbia-0 and C24 accessions (Rohde et al., 2004). In contrast, another experiment found that alfalfa hybrids had less cold tolerance based on electrical conductivity analysis (Kohel and Davis, 1960). Other results suggest additivity for these traits (Brummer et al., 2000; Daday and Greenham, 1960). The only quantitative trait loci (QTL) experiment to examine gene action found that winter injury was controlled by additive gene action, while partial dominance controlled fall growth and freezing injury (Brouwer et al., 2000).

Correlations between winter hardiness and autumn growth span from none to strong (Table I). The results of experiments, such as that of Schwab et al. (1996), in which cultivars from throughout the spectrum of winter hardiness were evaluated, show that a negative phenotypic correlation between autumn plant height and winter injury is evident. Similarly, genetic correlations of these traits were also high in an analysis of progeny from cultivars ranging widely in winter hardiness. In contrast, no genetic correlation was observed in a variable F_1 population derived from parents that were both semidormant (Brummer et al., 2000). This result,

Table I
Genetic (r_A) and Phenotypic (r_P) Correlations Between Winter Hardiness and Fall Dormancy Measured as Autumn Plant Growth

Germplasm	Traits correlated	r_A	r_P	Reference
F$_2$ progeny derived from dormant × nondormant crosses	Electrical resistance and winter yield		−0.38	Daday and Greenham, 1960
Dormant and nondormant plants and their F$_1$ and F$_2$ progenies	Winter survival and autumn height		−0.54	Kohel and Davis, 1960
15 cultivars with a range of fall dormancies	Winter injury and autumn height		0.94	Smith, 1961
F$_1$ and F$_2$ progeny from crosses among cultivars of all dormancy classes	Frost injury and growth at low temperature	−0.03 to 0.08	−0.19 to 0.02	Daday, 1964
14 F$_2$ progeny populations derived from dormant by moderately dormant crosses	Spring recovery and autumn height		0.08	Busbice and Wilsie, 1965
Six cultivars ranging from dormant to nondormant and their F$_1$ and S$_1$ progeny	Specific conductance and autumn height	0.91 to 1.20	0.28 to 0.54	Perry *et al.*, 1987
251 cultivars ranging widely in fall dormancy	Winter injury and autumn height		0.85	Schwab *et al.*, 1996
15 genotypes ranging from dormant to nondormant	Winter injury and autumn height		0.67	Brouwer *et al.*, 1998
Progeny population of dormant × nondormant hybrid backcrossed to dormant parent	Winter injury and autumn height		0.56	Brouwer *et al.*, 2000
F$_1$ population derived from a cross of two dormant genotypes	Winter injury and autumn height	−0.16	0.06	Brummer *et al.*, 2000
Hybrid F$_1$ populations from crosses of nine dormant to moderately dormant *M. sativa* and five dormant *M. falcata* genotypes	Winter injury and autumn height		0.29	Riday and Brummer, 2002b
Nondormant cultivars and derived populations with improved winterhardiness	Winter injury and autumn height		0.95	Weishaar *et al.*, 2005

coupled with similar findings by Busbice and Wilsie (1965) and Daday (1964) suggest that across a more constrained range of winter hardiness, tall plants in the autumn can be selected that also have superior winter hardiness.

The variation in results for heritability, gene action, and correlations arise from the variety and the breadth of the germplasm being evaluated, the environmental conditions to which the plants are subjected, and the method used to assay for the traits. A more rigorous evaluation of progeny from multiple genetic backgrounds across multiple locations would yield better information on genetic variance components, heritabilities, and importantly, genetic correlations among traits.

The problem faced by alfalfa breeders is not to simply increase winter hardiness—that is easily found in very northerly adapted germplasm—but to increase winter hardiness in high yielding alfalfa germplasm. Therefore, the critical question for plant breeders is the extent that winter hardiness and autumn growth result from pleiotropic effects of a set of genes controlling both traits or is determined by linkage due to past natural selection constraining populations to particular alleles. Most of the evidence suggests that the traits may be manipulated independently, at least to a degree, and that alfalfa breeders have the opportunity for developing improved winter hardy, high-yielding cultivars.

2.   Gain from Selection

Few experiments have been reported in which selection has been expressly applied for improved winter survival. Selection of hardy and nonhardy plants within cultivars resulted in populations that were hardy with short autumn height and nonhardy with tall autumn height (Smith, 1961). Plants selected from a cultivar trial in Alaska resulted in a very winter-hardy cultivar (Klebesadel, 1971). Neither of these experiments were actual recurrent selection programs.

Weishaar *et al.* (2005) have used three cycles of phenotypic recurrent selection for decreased winter injury to improve winter survival of four nondormant cultivars. Averaged across cultivars, winter injury measured in April decreased by two points on a five-point scale, from 4.4 to 2.4 in a linear fashion across the three cycles. In addition, the number of dead plants was sharply reduced by one cycle of selection, from 39% to 2% on average. This trial clearly showed that selection for improved winter survival could be effective in nondormant cultivars. However, concurrent with the decreased winter injury was a linear decrease in plant height. Biomass production in September showed a moderate increase across cycles, although November biomass production was depressed. Clearly, simple phenotypic selection

based on a visual score of winter injury was effective to consistently and significantly improve winter hardiness of nondormant populations.

Selection for altered autumn plant height was conducted in central California, a location without severe winter (Cunningham, *et al.*, 1998). Simple phenotypic recurrent selection for reduced height resulted in a population derived from the nondormant cultivar CUF101 with increased winter survival in Indiana, a location with moderate winter. Selection in cultivars with more dormancy also decreased autumn height but did not improve winter survival, which was already greater than 75%. Selection in the extremely nondormant cultivar Wadi Qurayat also led to reduced height, but it had no effect on survival, which was essentially zero. This result suggests that very nondormant germplasm do not have the ability to initiate any acclimation response, while all other germplasm can initiate the response and selection can improve allele frequencies at loci that modulate the intensity of response.

A tightly controlled, laboratory-based protocol to select for one or more components of winter hardiness avoids the variability of winter stress, and perhaps, results in a more rapid selection response than typical field-based approaches. A laboratory screen to select for freezing tolerance has been applied to several alfalfa populations for up to seven cycles of recurrent phenotypic selection, and gain in winter survival has been impressive (Castonguay *et al.*, 2005; Nadeau *et al.*, 2002). In the protocol, young alfalfa plants are acclimated at low-nonfreezing temperatures and then placed in freezing temperatures that are successively lowered over a period of time until an expected LT_{50} temperature is reached. The key to this selection protocol is that it is highly controlled, enabling the stress to be uniformly applied. Multiple cycles are possible per year. The most vigorous survivors are selected and intercrossed to begin the next cycle of selection. To our knowledge, this is the only nonfield based selection protocol that has successfully resulted in enhanced winter hardiness in the field (Fig. 7).

3. Problems and Challenges

The important point of selection for winter hardiness is that the germplasm selected must also be high yielding. Thus, selection solely for winter hardiness without concurrent (or successive) selection for yield will not result in useful germplasm. To our knowledge, no experiments in which concurrent selection for both increased autumn plant height (or, more saliently, increased autumn biomass production) and decreased winter injury have been conducted. The results of the Weishaar *et al.* (2005) and Cunningham *et al.* (1998) experiments strongly suggest that selecting simultaneously for both traits is necessary to prevent an undesirable decline in the other, and to
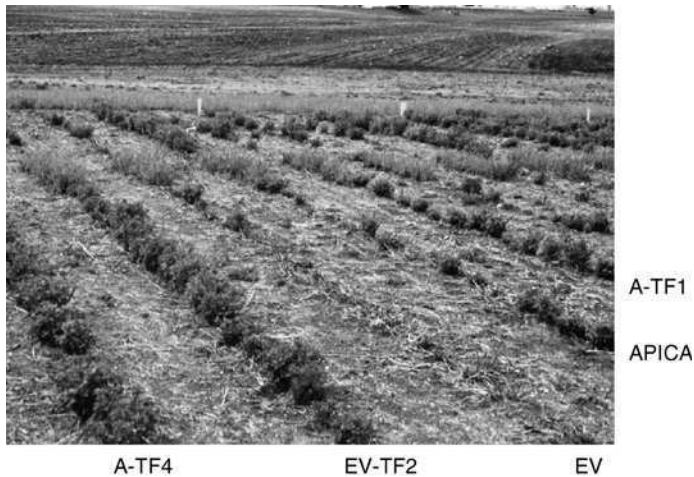
Figure 7 Photo depicting winter survival of alfalfa populations selected for improved freezing tolerance after exposure to a severe winter near Québec City. Populations potentially more tolerant to freezing (TF) were derived from the cultivar Apica (A-TF) and Evolution (EV-TF) using an approach developed by Nadeau *et al.* (2002). The number of cycles of recurrent selection cycles is indicated after the TF designation. The marked improvement in survival due to selection from within both genetic backgrounds is clearly visible.

some extent, commercial alfalfa breeding companies may have been selecting for both of these traits. The incorporation of a laboratory selection method, such as that of Nadeau *et al.* (2002), to augment field selection and speed the population improvement program, appears to be sensible.

The development of a selection index for cold tolerance, yield, and other traits could be developed to ensure that all important traits are being selected concurrently. However, it would require that either clonal or progeny evaluations were conducted, something not commonly done in many breeding programs. A move toward progeny testing so that progeny families could be evaluated for all the desired traits and selections based on those results should be adopted in alfalfa breeding.

One potential means of improving yield is through capturing heterosis (Brummer, 1999). Several experiments have shown that the progeny of modern elite cultivars and certain wild or semi-improved *M. sativa* subsp. *falcata* populations produce high biomass yield (Riday and Brummer, 2002a, 2005). However, in order for commercially viable cultivars to be developed from these crosses, the negative attributes of *falcata* germplasm, notably slower regrowth and early autumn dormancy (Riday and Brummer, 2002b) need to be mitigated. A selection program for both yield and autumn height may very well be useful.

Finally, a forward-looking program to improve yield and winter hardiness could be achieved by a sequential ratcheting of both traits by incorporating marginally less dormant material into the breeding program (Brummer *et al.*, 2000). This program would take advantage of the fact that within restricted ranges of germplasm, the phenotypic and/or genetic correlation between traits is less marked than when compared across the whole range.

C. Marker-Assisted Selection

1. QTL Mapping and Genetic Control of Freezing Tolerance

The possibility of improving both winter survival and autumn biomass production may be realized more quickly if genomic regions controlling one or both traits could be targeted by marker-assisted selection. Using markers in perennial crops has the substantial potential benefit of decreasing years per evaluation, thereby greatly enhancing genetic gain. In this respect, markers have much more potential benefit than in the annual crops in which a cycle of selection can already be completed in a year. Two experiments have been conducted in alfalfa to assess winter injury, autumn growth, and related traits (Alarcón-Zúñiga, 2003; Alarcón-Zúñiga *et al.*, 2004; Brouwer *et al.*, 2000). The Brouwer *et al.* (2000) experiment included two backcross populations derived from a winterhardy by nonwinter-hardy cross. Autumn growth (height), winter injury, and freezing injury assessed by electrical conductivity were measured and molecular markers were found to be associated with all of them. Multiple regression models were constructed that could explain between 6% and 52% of the phenotypic variation in the population for these traits in any given year or for the average across 2 years. The autumn growth QTL, however, could be related to differences in plant vigor and may not be related to dormancy *per se*, particularly in the population derived from the backcross to the nondormant parent (Brouwer *et al.*, 2000).

In the experiment of Alarcón-Zúñiga (2003), a tetraploid F_1 population derived from two hardy genotypes, one *M. sativa* subsp. *falcata* and the other *M. sativa* subsp. *sativa*, was scored for winter injury, autumn growth, and a suite of biochemical compounds in taproots that are typically associated with winter survival. Using near infrared reflectance spectrophotometry (NIRS), starch, crude and soluble protein, amino-N, TNC, sugars (glucose, fructose, and Suc), and fatty acids (oleic, palmitic, stearic, linoleic, and linolenic) were analyzed and genetically mapped. Protein and amino-N showed moderate positive genetic correlations with winter injury, but most of the remaining compounds were not correlated with injury. All traits could be associated with one or more QTL loci that could explain as much

as 69% of the phenotypic variation, with individual QTL alleles explaining from 4% to 39% of the variability (Brummer *et al.*, 2005). Some of the QTL for individual compounds appear to be in regions containing loci for autumn growth or winter survival, although much more work needs to be done to confirm those locations.

Importantly, in both studies, at least some loci affecting autumn growth and winter injury appear to be acting only on a single trait, giving further support to the concept that these traits can be selected independently, at least to an extent. However, both experiments found QTL × environment inter-actions, with some loci controlling a trait in 2 years but others being specific to a particular year. Many QTL experiments in other crops and for other complex traits have identified environment-specific QTLs (e.g., soybean protein and oil concentration; Brummer *et al.*, 1997). A simple explanation for this is simply that the population sizes are small and random error can affect detection of QTL, so that even if a locus is actually involved in winter hardiness each year, its effect may change in magnitude and not be detected in one of them. However, QTL × environment interactions could also be due to the complexity of the winter hardiness trait itself. In any one winter, different mechanisms may contribute more or less to tolerance—for exam-ple, the presence of freeze–thaw cycles in 1 year may be absent in another. Therefore, to some extent, the QTL detected will be a function of the winter environment in which the plants are grown and may not represent universally important loci.

If the second hypothesis is correct, that different loci are important to winter survival or to autumn growth to different degrees in each year, then the use of marker-assisted selection will be difficult. The major advan-tage of evaluating plants in the field is that even though we do not know which genetic loci are involved in winter survival, we can easily select for plants with the appropriate combination of alleles simply by assessing survival.

Marker-assisted selection will be less beneficial to breeding programs than marker-only selection. If the markers could be used to select plants in the greenhouse, it would accelerate selection progress significantly more than if they only augment field-based phenotypic data. A marker screen would need to be more effective than a lab-based selection method like that of Nadeau *et al.* (2002), however, to be useful to breeders. Perhaps the most benefit from markers could be garnered by using them to select against likely undesirable genotypes so that the material evaluated in the field is skewed toward better material. This would have the effect of making pheno-typic evaluations focus on more good families and hence would enhance genetic gain.

Using markers for QTL in a recurrent selection program poses several dilemmas for the plant breeder. First, most QTL experiments are conducted

in simple biparental cross-populations rather than within the entire breeding population itself. Thus, the loci and alleles of greatest importance within the population may well not be identified in the selected parents. One way to circumvent this problem could be to pursue an association-mapping approach (Skøt *et al.*, 2005), in which the members of the breeding population are genotyped throughout the genome and evaluated for phenotype. Complexities of tetraploidy and incomplete genotype information need to be addressed, and some level of understanding about the amount of linkage disequilibrium (LD) present within breeding populations is needed before this approach can be clearly shown to be useful.

Second, the measurement of phenotype presents a particular problem. Typically, breeding programs focus on individual plant evaluations, rather than the evaluation of progeny families. Given that family evaluation would help in the development of an index selection for multiple traits, as described above, moving in that direction would also enable better genetic mapping programs to be developed and consequently, would enhance the ability to use markers in the selection program. Ironically, better phenotypic data would increase heritabilities, making conventional selection much more effective, and hence markers would have less ability to improve selection.

Finally, comparative mapping of winter hardiness and related traits in other forage legumes, other crops, and in model systems may help refine our ability to identify critical loci and to use them in breeding programs.

2. Model Genetic Systems and Improvement
of Freezing Tolerance of Alfalfa

Apart from the large number of ESTs (>190,000) that have been sequenced from *Medicago truncatula*, a model species for the study of legume genomics (May, 2004), there are relatively few alfalfa cDNA sequences reported in public databases (Volenec *et al.*, 2002). Its small genome, amenability to transformation and large-scale assessment of gene function are attributes that warranted the choice of *M. truncatula* as a model species for the study of the legume genome (Cook, 1999). A *Medicago* genome array with ≈60,000 probes prepared mostly with *M. truncatula* EST/gene probes and including a small number (≈2000) *M. sativa* EST/mRNA has been released by Affymetrix (http://www.affymetrix.com). This will provide the plant scientists community with a powerful gene discovery tool for the study of the genetic bases of many traits in legumes. A high degree of gene-order conservation (synteny) has been confirmed between *M. truncatula* and

M. sativa (Choi *et al.*, 2004). Comparative mapping between *M. sativa* and *M. truncatula* has been successfully used for surrogate cloning of a gene required for nodulation in the more easily tractable *M. truncatula* (Endre *et al.*, 2002). Despite such success, frequent exceptions to the conserved synteny resulting from local genic rearrangements have been documented and may interfere with the practical use of syntenic relationships between model and crop species for gene identification (Vision, 2005; Zhu *et al.*, 2005a).

When we consider the Mediterranean origin, annual growth habit and limited capacity to survive freezing temperatures of *M. truncatula*, questions can be raised whether its genome arrays will contribute to our understanding of the genetics of winter hardiness in cultivated alfalfa. Will the lack of *M. sativa* sequence affect our long-term capacity to develop molecular approaches to improve freezing tolerance? Could the *M. truncatula* genome project provide a useful source of genes for the improvement of cold tolerance of alfalfa? Gene expression data that profile the response of the freezing-intolerant *M. truncatula* will undoubtedly help identify molecular features of the plant response to low temperature as the *Arabidopsis* model system has already done (Thomashow, 2001). However, critical genes and alleles that affect traits like freezing tolerance and perennial growth may be completely absent or exist in nonfunctional forms in the genome of these model species and may only be found in species that possess winter survival potential (Bressan *et al.*, 2001; Inan *et al.*, 2004). Some of the stress-induced genes found in *Thellungiella salsuginea*, a close relative of *Arabidopsis*, had low-sequence similarity with those found in GenBank suggesting unique functions that may contribute to *Thellungiella's* high tolerance to environmental stresses (Wong *et al.*, 2005). This clearly indicates that EST collections will be essential for species like alfalfa for which whole-genome sequence data will not be available in the foreseeable future. Thus, scarcity of DNA sequence information derived from *M. sativa* genomes could be a major impediment to the future development of marker-assisted selection (MAS) approach for this economically important crop. In that context, Canadian researchers have initiated an alfalfa genomic project targeted at the study of the transcriptome of cold-acclimated tetraploid alfalfa. Results from this project have been assembled into a searchable database that regroups the sequences of ~10,000 alfalfa cDNAs (Gagné *et al.*, 2004). These ESTs were obtained from plants that were extensively cold acclimated in order to identify genes that mechanistically confer protection against freezing. Such emerging EST collections from crop plants combined with a broad range of genotypes altered for the trait of interest will significantly increase the likelihood of uncovering key adaptive genes associated with superior winter hardiness (Fig. 8).
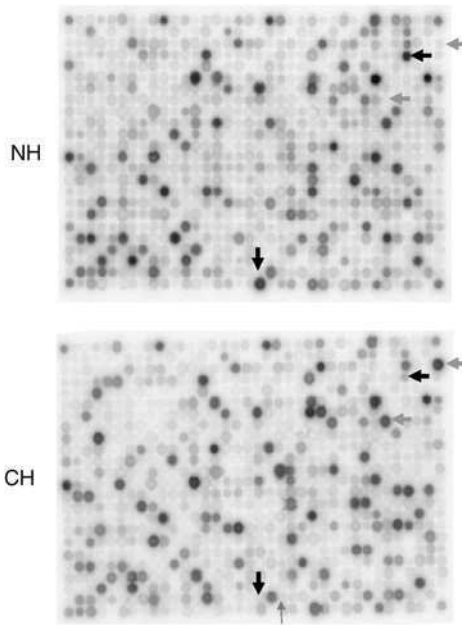
Figure 8 Macroarray analysis of changes in the expression alfalfa expressed sequence tags (ESTs) hybridized with cDNAs synthesized from mRNA purified from nonhardened (NH) and cold-hardened (CH) plants of alfalfa. Dark arrows indicate genes whose expression is downregulated and gray arrows indicate genes whose expression is upregulated at low temperature. Results from Desgagnés, R., Gagné, D., Castonguay, Y., and Laberge, S. (unpublished).

3. Identification of DNA Variants Associated with Freezing Tolerance

Our ability to routinely incorporate genes conferring superior freezing tolerance in cultivars of high-agronomic value will depend on the development of efficient introgression methodologies. New approaches are emerging, but their successful application will depend on a detailed knowledge of the genetic bases of superior freezing tolerance. The huge amount of published reports on the physiological, biochemical, and molecular changes that occur during cold acclimation of plants has thus far failed to provide an effective molecular approach to improve freezing tolerance in plants (Zhu *et al.*, 2005). One specific challenge that has arisen is how to use the information gained from genomics studies of model and crop species to produce new commercial plant varieties (Zhang *et al.*, 2004a). Many papers have reported positive results in the improvement of cold tolerance when plants were engineered with genes encoding transcriptional factors or key enzymes in the synthesis of cryoprotectants or protective proteins (Iba, 2002;

Zhang *et al.*, 2004a). However, spurious results are frequently obtained as a result of the fragmentary understanding of factors controlling transgene expression and the fact that a single gene change may not invoke a large increase in freezing tolerance.

The identification of genes having major effects on freezing tolerance of alfalfa is difficult due to its large genome and the complex genetics of populations of heterozygous genotypes (Brummer, 2004). Alternative approaches relying on candidate gene diversity across natural populations to uncover polymorphisms that correlate with phenotypic variation have emerged (Buckler IV and Thornsberry, 2002). Trait-allele association studies are rapidly developing and are bound to provide a much better understanding of the allelic diversity of breeding populations (Rafalski, 2002). Techniques like LD-based association tests (Gupta *et al.*, 2005) combined with high throughput assessment of DNA polymorphisms within coding and promoter regions of candidate genes could, significantly contribute to the identification of genes of adaptive value for complex traits like freezing tolerance. However, lack of genome-wide annotation of nucleotide diversity will limit the application of this approach for the study of population genetics in *M. sativa*. Bulk segregant analysis (BSA) is another very efficient gene discovery approach that has been successfully used to find unique genetic polymorphisms associated with stress tolerance in plants (Michelmore *et al.*, 1991; Quarrie *et al.*, 1999). Candidate genes potentially associated with freezing tolerance in alfalfa have been tested using restriction fragment length polymorphisms for differences in allele frequency using populations selectively improved for their freezing tolerance (Castonguay *et al.*, 2005). Polymorphisms that intensified with the number of selection cycles were uncovered for a number of candidate genes, including homologues of GaS and dehydrin. There is high likelihood that these polymorphic genes are linked to QTLs controlling freezing tolerance considering that these populations were derived from a common genetic background and that selection was solely targeted toward the improvement of that trait. Further research will, however, be required to determine the overall contribution of each polymorphic change to the determination of freezing tolerance in order to establish their usefulness as markers in breeding programs. Vision (2005) noted that the clearest functional links between gene polymorphisms and a particular phenotype were not derived from large-scale comparative sequencing analyses but rather from the availability of unique phenotypes. Results by Castonguay *et al.* (2005) illustrate that the availability of contrasted genetic material combined with stress-relevant DNA sequences could allow the identification of unique genetic polymorphisms associated with cold tolerance in a species with complex genomes like alfalfa.

D.   CONCEPTUAL APPROACH TO THE GENETIC CONTROL
OF FREEZING TOLERANCE IN ALFALFA

Our understanding of the morphological, physiological, and molecular bases of cold acclimation of alfalfa is more comprehensive than ever, and we now have a good grasp of the plant ideotype and metabolic traits that are associated with adaptation to winter. The challenge that scientists are now facing is how to use this information to devise new selection technologies for the improvement of cold tolerance of alfalfa and improve management practices to optimize winter survival. Although morphological or biochemical markers have been previously proposed for the development of winter-hardy cultivars, they were found to be either unsatisfactory or unreliable. Decline in seasonal productivity in cultivars developed on the basis of greater fall dormancy and the instability of biochemical markers due to $G \times E$ interactions preclude the use of these selection approaches in the development of cultivars of high-agronomic value. On the other hand, the development of more stable and precise MAS approaches will necessitate a better understanding of the genetic bases of superior cold tolerance.

A number of research reports cited earlier in this review have documented the fact that variation in the cold-acclimation response between cultivars of alfalfa is associated with differences in the levels of COR genes expression. Studies have provided evidence that differential gene expression within and between populations is a relatively common phenomenon that results from heritable allelic differences that might be physiologically significant (Knight, 2004). These allele-specific effects on gene expression are highly context specific and depend on cell type, developmental stage, and environmental conditions. Naturally occurring allelic diversity is thought to be a determinant factor of phenotypic variation in plants and may be an underlying mechanisms of QTL variation (Buckler and Thornsberry, 2002). Although genetic variation has been classically viewed in terms of coding-region polymorphisms that could alter protein structure and function, allele-specific differences in gene expression have been linked to *cis*-acting sequence variation emphasizing the equal importance of polymorphism in noncoding DNA in the determination of phenotypic diversity (Cowles *et al.*, 2002). Guo *et al.* (2004b) looked at allelic variation of gene expression in maize hybrids and observed that 73% of tested genes showed allele-specific regulation in response to environmental stress suggesting the possibility of an unequal function of parental alleles in conferring environmental fitness. Such results emphasize the fact that information on allele-specific sequences derived from genetically relevant phenotypes will be a prerequisite to future progress in the understanding of the genetics bases of cold tolerance in alfalfa.

Based on the understanding that freezing tolerance is a multigenic trait involving genes with variable effects, we propose a conceptual framework

for the genetic determination of freezing tolerance in alfalfa (Fig. 9). The existence of two discontinuous classes for field survival and cryoprotective soluble sugar accumulation among populations of contrasting fall dormancy (Fig. 10) suggests that, although autumn growth habit behaves as a quantitative trait involving many genes, its impact on the acquisition of cold tolerance could be mediated via of a smaller number of genes with major effects. These genes would provide a link between fall dormancy and molecular changes at low temperature and could be responsible for the occurrence of the two distinct classes of winter survival among the continuous gradient of fall dormancy responses (Fig. 10). It has been observed that constitutive overexpression of the CBF regulon in *Arabidopsis* can have effects akin to fall dormancy response in alfalfa by slowing growth and reducing plant height (Gilmour *et al.*, 2000). In addition to the impact of the genetic background, the expression of these regulatory genes is potentially affected by ontogenic and environmental factors known to influence autumn growth of alfalfa such as declining photoperiod, the developmental stage of the plant, autumn cutting, and soil fertility. These regulatory genes subsequently have a downstream impact on the expression of a large number of allelic variants with variable contribution to the determination of the level of freezing tolerance. The fact that cultivars of contrasting winterhardiness are able to cold acclimate, albeit to varying levels, indicates that differences
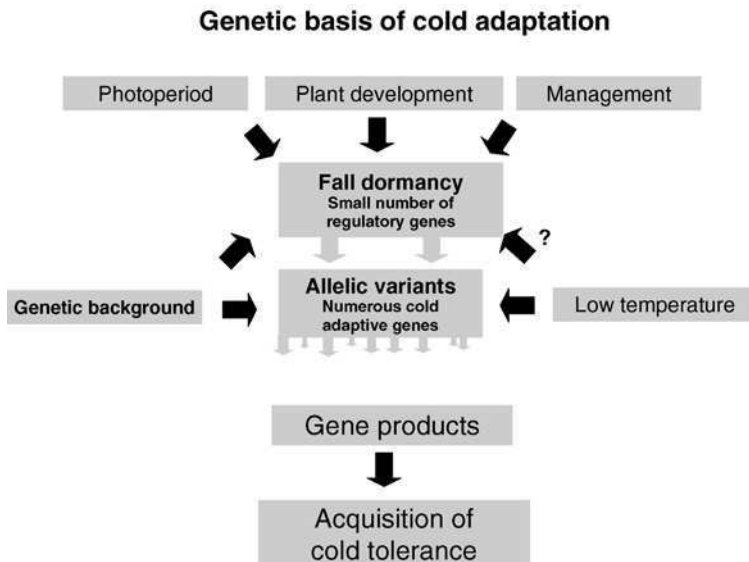
Figure 9 Conceptual model for the genetic bases of cold tolerance in alfalfa.
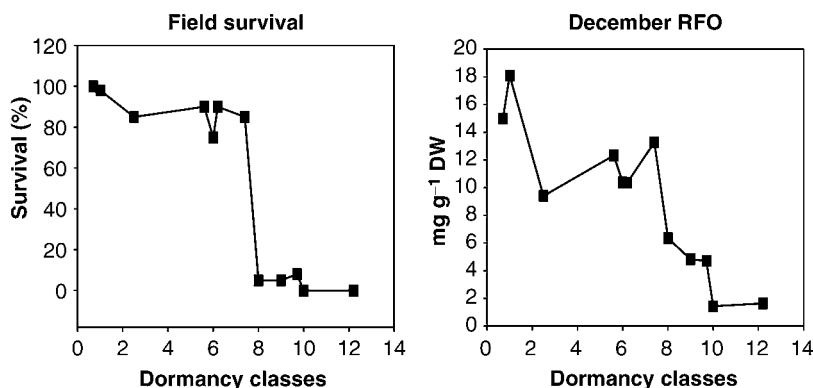
Figure 10 Relationship between field survival and raffinose family oligosaccharide (RFO) accumulation in taproots of fall hardened alfalfa and dormancy rating of fall dormancy selections. Note the two discontinuous classes of field survival and taproot RFO concentrations that occurs with the decline in fall dormancy (increasing fall dormancy class). Adapted from Cunningham *et al.* (2003).

in freezing tolerance could be under the control of a limited number of genes with major effects whose products have unique adaptive values with regard to low-temperature tolerance. The level of expression of these genes will be affected by the allelic forms that are present (genetic background) and sustained exposure to low temperature. DNA variants that show optimal level of expression or that have largest effects on freezing tolerance will be good candidates for the development of MAS technologies.

REFERENCES

Ain-Lhout, F., Zunzunegui, F. A., Diaz Barradas, M. C., Tirado, R., Clavijio, A., and Garcia Novo, F. (2001). Comparison of proline accumulation of two Mediterranean shrubs subjected to natural and experimental water deficit. *Plant Soil* **230**, 175–183.

Alarcón-Zúñiga, B. (2003). Genetic basis of autumn growth and winter hardiness in alfalfa. Ph.D. Dissertation. Iowa State University, IA.

Alarcón-Zúñiga, B., Brummer, E. C., Scott, P., Moore, K., and Luth, D. (2004). Quantitative trait loci mapping of winter hardiness metabolites in autotetraploid alfalfa. *In* "Molecular Breeding in Forage and Turf" (A. Hopkins, *et al.*, Eds.), pp. 97–104. Kluwer, Dordrecht, The Netherlands.

Anchordoguy, T. J., Rudolph, A. S., Carpenter, J. F., and Crowe, J. H. (1987). Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **24**, 324–331.

Antikainen, M., and Griffith, M. (1997). Antifreeze accumulation in freezing-tolerant cereals. *Physiol. Plant* **99**, 423–432.

Armengaud, P., Thiery, L., Buhot, N., Grenier-de March, G., and Savouré, A. (2004). Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. *Physiol. Plant* **120**, 442–450.

Bachman, M., Matile, P., and Keller, F. (1994). Metabolism of the raffinose family of oligosaccharides in leaves of *Ajuga reptans* L. *Plant Physiol.* **105**, 1335–1345.

Barnes, R. F., and Nelson, C. J. (2003). Forage and grasslands in a changing world. *In* "An Introduction to Grassland Agriculture" (R. F. Barnes, C. J. Nelson, M. Collins, and K. J. Moore, Eds.), pp. 3–23. Forages, Iowa State Press, Ames, IA.

Barnhart, S., Duffy, M., and Smith, D. (2004). Estimating cost of pasture and hay production. Ext. Bull. AG-96, Iowa State University, Ames, IA.

Bartels, D., and Sunkar, R. (2005). Drought and salt tolerance. *Crit. Rev. Plant Sci.* **24**, 23–58.

Battista, J. R., Park, M. J., and McLemore, A. E. (2001). Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes *Deinococcus radiodurans* R1 to desiccation. *Cryobiology* **43**, 133–139.

Bélanger, G., Rochette, P., Castonguay, Y., Bootsma, A., Mongrain, D., and Ryan, D. A. J. (2002). Climate change and winter survival of perennial forage crops in Eastern Canada. *Agron. J.* **94**, 1120–1130.

Belesky, D. P., and Fedders, J. M. (1997). Residue height influences stand dynamics of alfalfa grown on a shallow soil. *Agron. J.* **89**, 975–980.

Benoit, G. R., Fisher, K. D., and Bornstein, J. (1967). Alfalfa survival-indicator of sloping land drainage effectiveness. *Agron. J.* **59**, 444–447.

Berg, W. K., Cunningham, S. M., Brouder, S. M., Johnson, K. D., Joern, B. C., and Volenec, J. J. (2005). Influence of phosphorus and potassium fertilization on alfalfa yield and yield components. *Crop Sci.* **45**, 297–304.

Bertrand, A., Castonguay, Y., Nadeau, P., Laberge, S., Rochette, P., Michaud, R., Bélanger, G., and Benmoussa, M. (2001). Molecular and biochemical responses of perennial forage crops to oxygen deprivation at low temperature. *Plant Cell Environ.* **24**, 1085–1093.

Bertrand, A., Castonguay, Y., Nadeau, P., Laberge, S., Michaud, R., Bélanger, G., and Rochette, P. (2003). Oxygen deficiency affects carbohydrate reserves in overwintering forage crops. *J. Exp. Bot.* **54**, 1721–1730.

Bouyoucos, G. J., and McCool, M. M. (1928). The correct explanation for the heaving of soils, plants, and pavements. *Agron. J.* **20**, 480–491.

Bravo, L. A., and Griffith, M. (2005). Characterization of antifreeze activity in Antarctic plants. *J. Exp. Bot.* **56**, 1189–1196.

Bressan, R. A., Zhang, C., Zhang, H., Hasegawa, P. M., Bohnert, H. J., and Zhu, J.-K. (2001). Learning from the *Arabidopsis* experience. The next gene search paradigm. *Plant Physiol.* **127**, 1354–1360.

Brink, G. E., and Marten, G. C. (1989). Harvest management of alfalfa–nutrient yield vs. forage quality, and relationship to persistence. *J. Prod. Agric.* **2**, 32–36.

Brouwer, D. J., Duke, S. H., and Osborn, T. C. (1998). Comparison of seedlings and cuttings for evaluating winter hardiness in alfalfa. *Crop Sci.* **38**, 1704–1707.

Brouwer, D. J., Duke, S. H., and Osborn, T. C. (2000). Mapping genetic factors associated with winterhardiness, fall growth, and freezing injury in autotetraploid alfalfa. *Crop Sci.* **40**, 1387–1396.

Browne, J., Tunnacliffe, A., and Burnell, A. (2002). Plant desiccation gene found in nematode. *Nature* **416**, 38.

Brummer, E. C. (1999). Capturing heterosis in forage crop cultivar development. *Crop Sci.* **39**, 943–954.

Brummer, E. C. (2004). Applying genomics to alfalfa breeding programs. *Crop Sci.* **44**, 1904–1906.

Brummer, E. C., Graef, G. L., Orf, J., Wilcox, J. R., and Shoemaker, R. C. (1997). Mapping QTL for seed protein and oil content in eight soybean populations. *Crop Sci.* **37**, 370–378.

Brummer, E. C., Shah, M. M., and Luth, D. (2000). Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. *Crop Sci.* **40**, 971–977.

Brummer, E. C., Robins, J. G., Alarcón, Zúñiga, B., and Luth, D. (2005). Genetic mapping in tetraploid alfalfa: Results and prospects. *In* "Molecular Breeding for the Genetic Improvement of Forage Crops and Turf" (M. O. Humphreys, Ed.), p. 149. Wageningen Academic Publishers, Wageningen, The Netherlands.

Buckler, E., IV, and Thornsberry, J. M. (2002). Plant molecular diversity and application to genomics. *Curr. Opin. Plant Biol.* **5**, 107–111.

Bula, R. J., and Smith, D. (1954). Cold resistance and chemical composition in overwintering alfalfa, red clover, and sweetclover. *Agron. J.* **46**, 397–401.

Bula, R. J., Smith, D., and Hodgson, H. J. (1956). Cold resistance in alfalfa at two diverse latitudes. *Agron. J.* **48**, 153–156.

Buller, R. E., Pitner, J. B., and Ramirez, M. (1955). Behavior of alfalfa varieties in the valley of Mexico. *Agron. J.* **47**, 510–512.

Busbice, T. H., and Wilsie, C. P. (1965). Fall growth, winterhardiness, recovery after cutting and wilt resistance in F_2 progenies of Vernal x Dupuits alfalfa crosses. *Crop Sci.* **5**, 429–432.

Capell, T., Bassie, L., and Christou, P. (2004). Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proc. Natl. Acad. Sci.* **101**, 9909–9914.

Cary, E. E., Horner, G. M., and Mech, S. J. (1967). Relationship of tillage and fertilization to the yield of alfalfa on a Freeman silt loam. *Agron. J.* **59**, 165–168.

Castonguay, Y., and Nadeau, P. (1998). Enzymatic control of soluble carbohydrate accumulation in cold-acclimated crowns of alfalfa. *Crop Sci.* **38**, 1183–1189.

Castonguay, Y., Nadeau, P., and Laberge, S. (1993). Freezing tolerance and alteration of translatable mRNAs in alfalfa (*Medicago sativa* L.) hardened at subzero temperatures. *Plant Cell Physiol.* **34**, 31–38.

Castonguay, Y., Nadeau, P., Lechasseur, P., and Chouinard, L. (1995). Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winter hardiness. *Crop Sci.* **35**, 509–516.

Castonguay, Y., Laberge, S., Nadeau, P., and Vézina, L.-P. (1997a). Temperature and drought stress. *In* "Biotechnology and the Improvement of Forage Legumes" (B. D. McKersie and D. W. Brown, Eds.), pp. 175–202. CAB International.

Castonguay, Y., Nadeau, P., Laberge, S., and Vézina, L.-P. (1997b). Changes in gene expression in six alfalfa cultivars acclimated under winter hardening conditions. *Crop Sci.* **37**, 332–342.

Castonguay, Y., Laberge, S., Michaud, R., Nadeau, P., and Vézina, L.-P. (1998). Molecular approaches for the improvement of cold tolerance in alfalfa. *In* "Molecular and Cellular Technologies for Forage Management" (E. C. Brummer, Ed.), pp. 33–48. CSSA Special Publication no. 26.

Castonguay, Y., Cloutier, J., Laberge, S., Bertrand, A., and Michaud, R. (2005). A bulk segregant approach to identify genetic polymorphisms associated with cold tolerance in alfalfa. *In* "Cold Hardiness in Plants: Molecular Genetics, Cell Biology and Physiology" (T. H. H. Chen, M. Uemura, and S. Fujikawa, Eds.), pp. 88–102. CAB International, Wallingford, United Kingdom.

Chiang, H. H., and Dandekar, A. M. (1995). Regulation of proline accumulation in *Arabidopsis thaliana* (L.) Heynh during development and in response to desiccation. *Plant Cell Environ.* **18**, 1280–1290.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B. H., Hong, X., Agarwal, M., and Zhu, J. K. (2003). ICE1: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* **17**, 1043–1054.

Choi, D.-W., Rodriguez, E. M., and Close, T. J. (2002). Barley *Cbf3* gene identification, expression pattern, and map location. *Plant Physiol.* **129**, 1781–1787.

Choi, H. K., Kim, D., Uhm, T., Limpens, E., Lim, H., Mun, J. H., Kalo, P., Penmetsa, R. V., Seres, A., Kulikova, O., Roe, B. A., Bisseling, B. A., *et al.* (2004). A sequence-based genetic map of *Medicago truncatula* and comparison of marker colinearity with *M. sativa. Genetics* **166**, 1463–1502.

Close, T. J. (1996). Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiol. Plant.* **97**, 795–803.

Cloutier, Y., and Andrews, C. J. (1984). Efficiency of cold hardiness induction by desiccation stress in four winter cereals. *Plant Physiol.* **767**, 595–598.

Coleman, E. A., Bula, R. J., and Davis, R. L. (1966). Electrophoresis and immunological comparisons of soluble root proteins of *Medicago sativa* L. genotypes in cold hardened and nonhardened conditions. *Plant Physiol.* **41**, 1681–1685.

Cook, D., Fowler, S., Fiehn, O., and Thomashow, M. F. (2004). A prominent role for the CBF cold response pathway in configuring the low temperature metabolome in *Arabidopsis. Proc. Natl. Acad. Sci.* **101**, 15243–15248.

Cook, D. R. (1999). *Medicago truncatula*: A model in the making. *Curr. Opin. Plant Biol.* **2**, 301–304.

Couture, L., Dhont, C., Castonguay, Y., Nadeau, P., Chalifour, F. P., Drapeau, R., and Tremblay, G. (2002). Effect of the timing of the fall cut on fusarium root and crown rot in alfalfa. *Can. J. Plant Sci.* **82**, 621–624.

Cowles, C. R., Hirschhorn, J. N., Altshuler, D., and Lander, E. S. (2002). Detection of regulatory variation in mouse genes. *Nat. Genet.* **32**, 432–437.

Crews, T. E., and Peoples, M. B. (2004). Legume versus fertilizer sources of nitrogen: Ecological tradeoffs and human needs. *Agric. Ecosys. Environ.* **102**, 279–297.

Crowe, J. H., Hoesktra, F. A., and Crowe, L. M. (1992). Anhydrobiosis. *Ann. Rev. Physiol.* **54**, 579–599.

Cunningham, S. M., Volenec, J. J., and Teuber, L. R. (1998). Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. *Crop Sci.* **38**, 962–969.

Cunningham, S. M., Gana, J. A., Volenec, J. J., and Teuber, L. R. (2001). Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from ''Mesilla'' and "CUF 101" alfalfa. *Crop Sci.* **41**, 1091–1098.

Cunningham, S. M., Nadeau, P., Castonguay, Y., Laberge, S., and Volenec, J. J. (2003). Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting *Medicago sativa* germplasms. *Crop Sci.* **43**, 562–570.

Daday, H. (1964). Genetic relationship between cold hardiness and growth at low temperature in *Medicago sativa. Heredity* **19**, 173–179.

Daday, H., and Greenham, C. G. (1960). Genetic studies on cold hardiness in *Medicago sativa* L. *J. Hered.* **51**, 249–255.

Decker, A. M., Jr., and Ronningen, T. S. (1957). Heaving in forage stands and in bare ground. *Agron. J.* **49**, 412–415.

Dexter, S. T., Tottingham, W. E., and Graber, L. F. (1932). Investigations of the hardiness of plants by measurement of electrical conductivity. *Plant Physiol.* **7**, 63–72.

Dhanaraj, A. L., Slovin, J. P., and Rowland, L. J. (2004). Analysis of gene expression associated with cold acclimation in blueberry floral buds using expressed sequence tags. *Plant Sci.* **166**, 863–872.

Dhont, C., Castonguay, Y., Nadeau, P., Bélanger, G., and Chalifour, F.-P. (2002). Alfalfa root carbohydrates and regrowth potential in response to fall harvests. *Crop Sci.* **42**, 754–765.

Dhont, C., Castonguay, Y., Nadeau, P., Bélanger, G., and Chalifour, F.-P. (2003). Alfalfa root nitrogen reserves and regrowth potential in response to fall harvests. *Crop Sci.* **43**, 181–194.

Dhont, C., Castonguay, Y., Nadeau, P., Bélanger, G., Drapeau, R., Laberge, S., Avice, J.-C., and Chalifour, F.-P. (2006). Nitrogen reserves, spring regrowth, and winter survival of field-grown alfalfa (*Medicago sativa* L.) defoliated in the fall. *Ann. Bot.* **97**, 109–120.

Dörffling, K., Dörffling, H., and Lesselich, G. (1993). *In vitro*-selection and regeneration of hydroxyproline-resistant lines of winter wheat with increased proline content and increased frost tolerance. *J. Plant Physiol.* **142**, 222–225.

Doucet, C. J., Byass, L., Elias, L., Worral, D., Smallwood, M., and Bowles, D. J. (2000). Distribution and characterization of recrystallization inhibitor activity in plant and lichen species from UK and Maritime Antarctic. *Cryobiology* **40**, 218–227.

Downie, B., Gurusinghe, S., Dahal, P., Thacker, R. R., Snyder, J. C., Nonogaki, H., Yim, K., Fukanaga, K., Alvarado, V., and Bradford, K. J. (2003). Expression of a *galactinol synthase* gene in tomato seeds is up-regulated before maturation desiccation and again after imbibition whenever radicle protrusion is prevented. *Plant Physiol.* **131**, 1347–1359.

Drolet, G., Dumbroff, E. B., Legge, R. L., and Thompson, J. E. (1996). Radical scavenging properties of polyamines. *Phytochemistry* **25**, 367–371.

Duke, S. H., and Doehlert, D. (1981). Root respiration, nodulation, and enzyme activities in alfalfa during cold acclimation. *Crop Sci.* **21**, 489–495.

Endre, G., Kereszt, A., Kevei, Z., Mihaceas, S., Kaló, P., and Kiss, G. B. (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature* **41**, 962–966.

Ferrullo, J.-M., Vézina, L.-P., Rauil, J., Laberge, S., Nadeau, P., and Castonguay, Y. (1997). Differential accumulation of two glycine-rich proteins during cold-acclimation of alfalfa. *Plant Mol. Biol.* **33**, 625–633.

Fowler, S., and Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold responsive pathway. *Plant Cell* **14**, 1675–1690.

Freyman, S. (1969). Role of stubble in the survival of certain ice-covered forages. *Agron. J.* **61**, 105–107.

Freyman, S., and Brink, V. C. (1967). Nature of ice-sheet injury to alfalfa. *Agron. J.* **59**, 557–560.

Gagné, D., Desgagnés, R., Tinker, N., and Laberge, S. (2004). An alfalfa bioinformatic database for future discovery of important genes in alfalfa. 39th North American Alfalfa Improvement Conference, Quebec City, Canada [Online] Available: http://www.naaic.org/Meetings/National/index.html [Undated].

Gerloff, E. D., Stahmann, M. A., and Smith, D. (1967). Soluble proteins in alfalfa roots as related to cold hardiness. *Plant Physiol.* **42**, 895–899.

Gilmour, S. J., Hajela, R. K., and Thomashow, M. F. (1988). Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol.* **87**, 745–750.

Gilmour, S. J., Sebolt, A. M., Slazar, M. P., Everard, J. D., and Thomashow, M. F. (2000). Overexpression of the *Arabidopsis CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* **124**, 1854–1865.

Gilmour, S. J., Fowler, S., and Thomashow, M. F. (2004). *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.* **54**, 767–781.

Ginzberg, I., Stein, H., Kapulnik, Y., Szabados, L., Strizhov, N., Schell, J., Koncz, C., and Zilberstein, A. (1998). Isolation and characterization of two different cDNAs of delta1-pyrroline-5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress. *Plant Mol. Biol.* **38**, 755–764.

Grandfield, C. O. (1934). The effect of the time of cutting and of winter protection on the reduction of stands in Kansas common, Grimm, and Turkestan alfalfas. *Agron. J.* **26**, 179–188.

Griffith, M., and Yaish, M. F. W. (2004). Antifreeze proteins in overwintering plants: A tale of two activities. *Trends Plant Sci.* **9**, 399–405.

Griffith, M., Lumb, C., Wiseman, S. B., Wisniewski, M., Johnson, R. W., and Marangoni, A. G. (2005). Antifreeze proteins modify the freezing process in planta. *Plant Physiol.* **138**, 330–340.

Gross, H. D., Wilsie, C. P., and Pesek, J. (1958). Some responses of alfalfa varieties to fertilization and cutting treatments. *Agron. J.* **50**, 161–164.

Guo, Z.-J., Chen, X.-J., Wu, X.-L., Ling, J. Q., and Xu, P. (2004a). Overexpression of the AP2/EREBP transcription factor OPB1 enhances disease resistance and salt tolerance in tobacco. *Plant Mol. Biol.* **55**, 607–618.

Guo, M., Rupe, M. A., Zinselmeir, C., Habben, J., and Bowen, B. A. (2004b). Allelic variation of gene expression in maize hybrids. *Plant Cell* **16**, 1707–1716.

Gupta, P. K., Rustgi, S., and Kulwal, P. L. (2005). Linkage disequilibrium and association studies in higher plants: Present status and future prospects. *Plant Mol. Biol.* **57**, 461–485.

Guy, C. L., Niemi, K. J., and Brambl, R. (1985). Altered gene expression during cold acclimation of spinach. *Proc. Natl. Acad. Sci.* **81**, 3673–3677.

Guy, C. L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 187–223.

Haagenson, D. M., Cunningham, S. M., Joern, B. C., and Volenec, J. J. (2003). Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. *Crop Sci.* **43**, 1340–1348.

Handa, S., Handa, A., Hasegawa, P. M., and Bressan, R. A. (1986). Proline accumulation and adaptation of cultured plant cells to water stress. *Plant Physiol.* **80**, 938–945.

Handley, L. W., Pharr, D. M., and McFeeters, R. F. (1983). Relationship between galactinol synthase activity and sugar composition in leaves and seeds of several crop species. *J. Am. Soc. Hort. Sci.* **108**, 600–605.

Hara, M., Terashima, S., Fukaya, T., and Kuboi, T. (2003). Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. *Planta* **217**, 290–298.

Hara, M., Fujinaga, M., and Kuboi, T. (2004). Radical scavenging activity and oxidative modification of citrus dehydrin. *Plant Physiol. Biochem.* **42**, 657–662.

Hare, P. D., Cress, W. A., and Van Staden, J. (1998). Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* **21**, 535–553.

Heichel, G. H., and Henjum, K. I. (1990). Fall dormancy response of alfalfa investigated with reciprocal cleft grafts. *Crop Sci.* **30**, 1123–1127.

Hendershot, K. L., and Volenec, J. J. (1993). Taproot nitrogen accumulation and use in overwintering alfalfa. *J. Plant. Physiol.* **141**, 68–74.

Hien, D. T., Jacobs, M., Angenon, G., Hermans, C., Thu, T. T., Son, L. V., and Roosens, N. H. (2003). Proline accumulation and Δ^1-pyrroline-5-carboxylate synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. *Plant Sci.* **165**, 1059–1068.

Hincha, D. K., Neukamm, B., Sror, H. A. M., Sieg, F., Weckwarth, W., Rückels, M., Lullien-Pellerin, V., Schröder, W., and Schmitt, J. M. (2001). Cabbage cryoprotectin is a member of the nonspecific plant lipid transfer protein gene family. *Plant Physiol.* **125**, 835–846.

Hirt, H. (1999). Transcriptional upregulation of signaling pathways: More complex than anticipated? *Trends Plant Sci.* **4**, 7–8.

Hirt, H. (2000). MAP kinases in plant signal transduction. *In* "Map Kinases in Plant Signal Transduction" (H. Hirt, Ed.), pp. 1–10. Springer-Verlag, Berlin.

Hodgson, H. J. (1964). Effect of photoperiod on development of cold resistance in alfalfa. *Crop Sci.* **4**, 302–330.

Hong, Z., Lakkineri, K., Zhang, Z., and Verma, D. P. (2000). Removal of feedback inhibition of delta (1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.* **122**, 1129–1136.

Houde, M., Dallaire, S., N'Dong, D., and Sarhan, F. (2004). Overexpression of the acidic dehydrin WCOR410 improved freezing tolerance in transgenic strawberry leaves. *Plant Biotech. J.* **2**, 381–387.

Howe, G. T., and Brunner, A. M. (2005). An evolving approach to understanding plant adaptation. *New Phytol.* **167**, 1–5.

Hur, J., Jung, K.-H., Lee, C.-H., and An, G. (2004). Stress inducible *OsP5CS2* gene is essential for salt and cold tolerance in rice. *Plant Sci.* **167**, 417–426.

Iba, K. (2002). Acclimative response to temperature stress in higher plants: Approaches of genetic engineering for temperature tolerance. *Annu. Rev. Plant Biol.* **53**, 225–245.

Inan, G., Zhang, Q., Li, P. H., Wang, Z. L., Cao, Z. Y., Zang, H., Zhang, C. Q., Quist, T. M., Goodwin, S. M., Zhu, J. H., Shi, H. H., Damsz, H. H., *et al.* (2004). Salt cress. A halophyte and cryophyte *Arabidopsis* relative model system and its applicability to molecular genetic analyses of growth and development of extremophyles. *Plant Physiol.* **135**, 1718–1737.

Ismail, A. M., Hall, A. E., and Close, T. J. (1999). Allelic variation of a dehydrin gene cosegregates with chilling tolerance during seedling emergence. *Proc. Natl. Acad. Sci.* **96**, 13566–13570.

Jaglo, K. R., Kleff, S., Amundsen, K. L., Zhang, X., Haake, V., Zhang, J. Z., Deits, T., and Thomashow, M. F. (2001). Components of the *Arabidopsis* c-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.* **127**, 910–917.

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., and Thomashow, M. F. (1998). *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* **280**, 104–106.

Johnson, L. D., Marquez-Ortiz, J. J., Lamb, J. F. S., and Barnes, D. K. (1998). Root morphology of alfalfa plant introductions and cultivars. *Crop Sci.* **38**, 497–502.

Jonak, C., Kiergel, S., Ligterink, W., Barker, P. J., Huskinsson, N. S., and Hirt, H. (1996). Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci.* **93**, 11274–11279.

Jones, J. H., and Olsen, F. J. (1987). Alfalfa establishment and production on soils with different drainage characteristics. *Agron. J.* **79**, 152–154.

Junttila, O., and Robberecht, R. (1999). Ecological aspects of cold-adapted plants with a special emphasis on environmental control of cold hardening and dehardening. *In* "Cold-Adapted Organisms: Ecology, Physiology, Enzymology and Molecular Breeding" (R. Margesin and F. Schinner, Eds.), pp. 57–78. Springe-Verlag.

Kalengamaliro, N. E., Gana, J. A., Cunningham, S. M., and Volenec, J. J. (2000). Mechanisms regulating differential freezing tolerance of suspension cell cultures derived from contrasting alfalfa genotypes. *Plant Cell Tissue Org. Cult.* **61**, 143–151.

Kanneganti, V. R., Bland, W. L., and Undersander, D. J. (1998a). Modeling freezing injury in alfalfa to calculate forage yield: II. Model validation and example simulations. *Agron. J.* **90**, 698–704.

Kanneganti, V. R., Rotz, C. A., and Walgenbach, R. P. (1998b). Modeling freezing injury in alfalfa to calculate forage yield: I. model development and sensitivity analysis. *Agron. J.* **90**, 687–697.

Kasinathan, V., and Wingler, A. (2004). Effect of reduced arginine decarboxylase activity on salt tolerance and on polyamine formation during salt stress in *Arabidopsis thaliana*. *Physiol. Plant.* **121**, 101–107.

Kaur, N., and Gupta, A. K. (2005). Signal transduction pathways under abiotic stresses in plants. *Curr. Sci.* **88**, 1771–1780.

Kavi Kishor, P. B., Hong, Z., Miao, G.-H., Hu, C.-A. A., and Verma, D. P. S. (1995). Over-expression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* **108**, 1387–1394.

Kawczynski, W., and Dhindsa, R. S. (1996). Alfalfa nuclei contain cold-responsive phospho-proteins and accumulate heat-stable proteins during cold treatment of seedlings. *Plant Cell Physiol.* **37**, 1204–1210.

Kim, J. C., Lee, S. H., Cheong, Y. H., Yoo, C.-M., Lee, S. I., Chun, H. J., Yun, D.-J., Hong, J. C., Lee, S. Y., Lim, C. O., and Cho, M. J. (2001). A novel cold inducible zinc finger protein from soybean SCOF-1, enhances cold tolerance in transgenic plants. *Plant J.* **25**, 247–259.

Kim, Y. O., Kim, J. S., and Kang, H. (2005). Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana. Plant J.* **42**, 890–900.

Kinbacher, E. J. (1956). Resistance of seedlings to frost heaving injury. *Agron. J.* **48**, 166–170.

Kizis, D., Lumbreras, V., and Pagès, M. (2001). Role of AP2/EREB transcription factors in gene regulation during abiotic stress. *FEBS Lett.* **498**, 187–189.

Klebesadel, L. J. (1964). Modification of alfalfa root system by severing the tap root. *Agron. J.* **56**, 359–361.

Klebesadel, L. J. (1971). Selective modification of alfalfa toward acclimatization in a subarctic area of severe winter stress. *Crop Sci.* **11**, 609–614.

Klebesadel, L. J., and Brinsmade, J. C. (1966). Response of two alfalfas (*Medicago sativa* L. and *M. falcata* L.) to time and rate of potassium application in the subarctic. *Agron. J.* **58**, 545–549.

Klotke, J., Kopka, J., Gatzke, N., and Heyer, A. G. (2004). Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation- evidence for a role of raffinose in cold acclimation. *Plant Cell Environ.* **27**, 1395–1404.

Knight, H., and Knight, M. R. (2001). Abiotic stress signalling pathways: Specificity and cross-talk. *Trends Plant Sci.* **6**, 262–267.

Knight, J. C. (2004). Allele-specific gene expression uncovered. *Trends Genet.* **20**, 113–116.

Kohel, R. J., and Davis, R. L. (1960). The inheritance of cold resistance and its relation to fall growth in F2 and BC1 generations of alfalfa. *Agron. J.* **52**, 234–237.

Krasnuk, M., Witham, F. H., and Jung, G. A. (1978). Hydrolytic enzyme differences in cold-tolerant and cold-sensitive alfalfa. *Agron. J.* **70**, 597–605.

Kreps, J. A., Wu, Y., Chang, H.-S., Zhu, T., Wang, X., and Harper, J. F. (2002). Transcriptome changes for *Arabidopsis* in response to salt, osmotic and cold stress. *Plant Physiol.* **130**, 2129–2141.

Krol, E., Dziubińska, H., and Trebacz, K. (2004). Low-temperature-induced transmembrane potential changes in mesophyll cells of *Arabidopsis thaliana*, *Helianthus annuus* and *Vicia faba. Physiol. Plant.* **120**, 265–270.

Laberge, S., Castonguay, Y., and Vézina, L.-P. (1993). New cold- and drought-regulated gene from *Medicago sativa. Plant Physiol.* **101**, 1411–1412.

Larson, K. L., and Smith, D. (1963). Association of various morphological characters and seed germination with the winterhardiness of alfalfa. *Crop Sci.* **3**, 234–237.

Larson, K. L., and Smith, D. (1964). Reliability of various plant constituents and artificial freezing methods for determining the winterhardiness of alfalfa. *Crop Sci.* **4**, 413–417.

Leep, R. H., Andresen, J. A., and Jeranyama, P. (2001). Fall dormancy and snow depth effects on winterkill of alfalfa. *Agron. J.* **93**, 1142–1148.

Leopold, A. C. (1990). Coping with desiccation. *In* "Stress Responses in Plants: Adaptation and Acclimation Mechanisms" (J. G. Alscher and J. R. Cumming, Eds.), pp. 37–56. Wiley-Liss Inc., New York, NY.

Lim, C. C., Krebs, S. L., and Arora, R. (1999). A 25-kDa dehydrin associated with genotype- and age-dependent leaf freezing tolerance in Rhododendron: A genetic marker for cold hardiness? *Theor. Appl. Gen.* **99**, 912–920.

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Cri. Rev. Plant Sci.* **10**, 123–150.

Liu, J.-J., Krenz, D. C., Galvez, A. F., and de Lumen, B. O. (1998). Galactinol synthase (GS): Increased enzyme activity and levels of mRNA due to cold and desiccation. *Plant Sci.* **134**, 11–20.

Lopez, C. G., Banowetz, G. M., Peterson, J., and Kronstad, W. E. (2003). Dehydrin expression and drought tolerance in seven wheat cultivars. *Crop Sci.* **43**, 577–582.

Lutts, S., Kinet, J. M., and Bouharmont, J. (1999). Improvement of rice callus regeneration in presence of NaCl. *Plant Cell Tiss. Organ. Cult.* **57**, 3–11.

Marian, C. O., Krebs, S. L., and Arora, R. (2004). Dehydrin variability among rhododendron species: A 25-kDa dehydrin is conserved and associated with cold acclimation across diverse species. *New Phytol.* **161**, 773–780.

Marquez-Ortiz, J. J., Lamb, J. F. S., Johnson, L. D., Barnes, D. K., and Stucker, R. E. (1999). Heritability of crown traits in alfalfa. *Crop Sci.* **39**, 38–43.

Maryama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., Shimada, Y., Yoshida, S., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). Identification of cold-inducible down-stream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two micro-array systems. *Plant J.* **38**, 982–993.

Mattana, M., Biazzi, E., Consonni, R., Locatelli, F., Vannini, C., Provera, S., and Coraggio, I. (2005). Overexpression of *Osmyb4* enhances compatible solute accumulation and increases stress tolerance of *Arabidopsis thaliana*. *Physiol. Plant.* **125**, 212–223.

May, G. D. (2004). From model to crops: Integrated *Medicago* genomics for alfalfa improvement. *In* "Molecular Breeding of Forage and Turf" (A. Hopkins, Z. Y. Wang, R. Mian, M. Sledge, and R. E. Braker, Eds.), pp. 325–332. Kluwer Academics Publishers, Dordrecht.

Mazur, P. (2004). Principles of cryobiology. *In* "Life in the Frozen State" (B. J. Fuller, N. Lane, and E. E. Benson, Eds.), pp. 3–65. CRC Press, Boca Raton, USA.

McKenzie, J. S., Paquin, R., and Duke, S. H. (1988). Cold and heat tolerance. *In* "Alfalfa and Alfalfa Improvement" (A. A. Hanson, D. K. Barnes, and R. R. Hill, Jr., Eds.), pp. 259–302, Agron. Monogr. 29. ASA, CSSA, and SSSA, Madison, WI.

McKersie, B. D., Chen, Y., deBeus, M., Bowley, S. R., Bowler, C., Inzé, D., D'Halluin, K., and Botterman, J. (1993). Superoxide dismutase enhances tolerance of freezing stress in trans-genic alfalfa (*Medicago sativa* L.). *Plant Physiol.* **103**, 1150–1163.

McKersie, B. D., and Leshem, Y. Y. (1994). "Stress and Stress Coping in Cultivated Plants." Kluwer Academic Publishers, Dordrecht, The Netherlands.

McKersie, B. D., Bowley, S. R., Harjanto, E., and Leprince, O. (1996). Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* **111**, 1177–1181.

McKersie, B. D., Bowley, S. R., and Jones, K. S. (1999). Winter survival of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* **119**, 839–847.

Megee, C. R. (1935). A search for factors determining winter hardiness in alfalfa. *Agron. J.* **27**, 685–698.

Meuriot, F., Noquet, C., Avice, J. C., Volenec, J. J., Cunningham, S. M., Sors, T. G., Caillot, S., and Ourry, A. (2004). Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. *Physiol. Plant.* **120**, 113–123.

Michaud, R., Lehman, W. F., and Rumbaugh, M. D. (1988). World distribution and historical development. *In* Alfalfa and Alfalfa Improvement (A. A. Hanson, D. K. Barnes, and R. R. Hill, Jr., Eds.), pp. 25–91. ASA, CSSA, and SSSA, Madison, WI Agron. Monogr. 29.

Michaud, R., Richard, C., Willemot, C., and Gasser, H. (1983). Apica alfalfa. *Can. J. Plant Sci.* **63**, 547–549.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA* **88**, 9828–9832.

Mohapatra, S. S., Poole, R. J., and Dhindsa, R. S. (1987a). Changes in protein patterns and translatable messenger RNA populations during cold-acclimation of alfalfa. *Plant Physiol.* **84**, 1172–1176.

Mohapatra, S. S., Poole, R. J., and Dhindsa, R. S. (1987b). Cold acclimation, freezing tolerance and protein synthesis in alfalfa (*Medicago sativa* L. cv. Saranac). *J. Exp. Bot.* **38**, 697–703.

Mohapatra, S. S., Poole, R. J., and Dhindsa, R. S. (1988a). Alterations in membrane protein-profile during cold treatment of alfalfa. *Plant Physiol.* **86**, 1005–1007.

Mohapatra, S. S., Poole, R. J., and Dhindsa, R. S. (1988b). Abscisic acid-regulated gene expression in relation to freezing tolerance of alfalfa. *Plant Physiol.* **87**, 468–473.

Mohapatra, S. S., Wolfraim, L., Poole, R. J., and Dhindsa, R. S. (1989). Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of alfalfa. *Plant Physiol.* **89**, 375–380.

Monroy, A. F., and Dhindsa, R. S. (1995). Low temperature-signal transduction: Induction of cold aclimmation-specific genes of alfalfa by calcium at 25°C. *Plant Cell* **7**, 321–331.

Monroy, A. F., Sarhan, F., and Dhindsa, R. S. (1993a). Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. Evidence for a role for calcium. *Plant Physiol.* **102**, 1227–1235.

Monroy, A. F., Castonguay, Y., Laberge, S., Sarhan, F., Vézina, L. P., and Dhindsa, R. S. (1993b). A new cold-induced alfalfa gene is associated with enhanced hardening at subzero temperature. *Plant Physiol.* **102**, 873–879.

Monroy, A. F., Sangwan, V., and Dhindsa, R. S. (1998). Low temperature signal transduction during cold acclimation: Protein phosphatase 2A as an early target for cold-inactivation. *Plant J.* **13**, 653–660.

Mukhopaday, A., Vij, S., and Tyagi, A. K. (2004). Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci.* **101**, 6309–6314.

Nadeau, P., Delaney, S., and Chouinard, L. (1987). Effects of cold hardening on the regulation of polyamine levels in wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa* L.). *Plant Physiol.* **84**, 73–77.

Nadeau, P., and Paquin, R. (1988). Evolution of polyamines in winter cereal and forage species under field cold acclimation in Quebec. *Can. J. Plant Sci.* **68**, 449–456.

Nadeau, P., Castonguay, Y., Laberge, S., and Michaud, R. (2002). Characteristics of alfalfa populations selected for improved freezing tolerance. *In* 38th North American Alfalfa Improvement Conference, Sacramento, CA, USA, [Online] Available: http://www.naaic.org/Meetings/National/index.html [Undated].

Narusuka, Y., Narusuka, M., Seki, M., Umezawa, T., Ishida, J., Nakajima, M., Enju, A., and Shinozaki, K. (2004). Crosstalk in the responses to abiotic and biotic stresses in *Arabidopsis*: Analysis of gene expression in *cytochrome P450* gene superfamily by cDNA microarray. *Plant Mol. Biol.* **55**, 327–342.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett.* **461**, 205–210.

Nogueira, F. T. S., Schlögl, P. S., Camargo, S. R., Fernandez, J. H., De Rosa, J. H., Jr., Pompermayer, P., and Arruda, P. (2005). SsNAC23, a member of the NAC protein family, is associated with cold, herbivory and water stress in sugarcane. *Plant Sci.* **169**, 93–106.

Novillo, F., Alonso, J. M., Ecker, J. R., and Salinas, J. (2004). CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* **101**, 3985–3990.

Oliver, A. E., Leprince, O., Wolkers, W. F., Hincha, D. K., Heyer, A. G., and Crowe, J. H. (2001). Non-disaccharide-based mechanisms of protection during drying. *Cryobiology* **43**, 151–167.

Orr, W., Singh, J., and Brown, D. C. W. (1985). Induction of freezing tolerance in alfalfa cell suspension cultures. *Plant Cell Rep.* **4**, 15–18.

Örvar, B. L., Sandwan, V., Omann, F., and Dhindsa, R. S. (2000). Early steps in cold sensing by plant cells: The role of actin cytoskeleton and membrane fluidity. *Plant J.* **23**, 785–794.

Panikulangara, T. J., Eggers-Schumacher, G., Wunderlich, M., Stransky, H., and Schöffl, F. (2004). *Galactinol synthase*1. A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in *Arabidopsis*. *Plant Physiol.* **136**, 3148–3158.

Paquin, R. (1984). Influence of the environment on cold hardening and winter survival of forage plants and cereals; proline as a metabolic marker of hardening. *In* "Being Alive on Land" (N. S. Margaris, M. Arianoustou-Faraggitali, and W. C. Oechel, Eds.), pp. 137–154. Kluwer Academic Publishers Group, Boston.

Paquin, R., and Lechasseur, P. (1982). Acclimatation naturelle de la luzerne (*Medicago sativa* Pers.) au froid. II. Variations de la teneur en sucres totaux des feuilles et des collets. *Acta Oecologica/Oecologia Plantarum* **3**, 27–38.

Park, B.-J., Liu, Z., Kanno, A., and Kameya, T. (2005). Increased tolerance to salt- and water-deficit stress in transgenic lettuce (*Lactuca sativa* L.) by constitutive expression of LEA. *Plant Growth Regul.* **45**, 165–171.

Park, C. J., Park, C. B., Hong, S. S., Lee, H. S., Lee, S. Y., and Kim, S. C. (2000). Characterization and cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, *Capsella bursa-pastoris*. *Plant Mol. Biol.* **44**, 187–197.

Park, S. H., Jun, S.-S., An, G., Hong, Y.-N., and Park, M. C. (2003). A comparative study on the protective role of trehalose and LEA proteins against abiotic stresses in transgenic Chinese cabbage (*Brassica campestris*) overexpressing *CaLea* and *otsA*. *J. Plant Biol.* **46**, 277–286.

Pearce, R. S. (1988). Extracellular ice and cell shape in frost-stressed cereal leaves: A low temperature scanning electron microscopy study. *Planta* **175**, 313–324.

Pearce, R. S. (1999). Molecular analysis of acclimation to cold. *Plant Growth Regul.* **29**, 47–76.

Pearce, R. S. (2004). Adaptation of higher plants to freezing. *In* "Life in the Frozen State" (B. J. Fuller, N. Lane, and E. E. Benson, Eds.), pp. 171–203. CRC Press, Boca Raton, USA.

Pennycooke, J. C., Jones, M. L., and Stushnoff, C. (2003). Down-regulating α-galactosidase enhances freezing tolerance in transgenic petunia. *Plant Physiol.* **133**, 901–909.

Pennycooke, J. C., Vepachedu, R., and Stushnoff, C. (2004). Expression of an α-galactosidase gene in petunia is upregulated during low-temperature deacclimation. *J. Am. Soc. Hort. Sci.* **129**, 491–496.

Perfect, E., Miller, R. D., and Burton, B. (1987). Root morphology and vigor effects on winter heaving of established alfalfa. *Agron. J.* **79**, 1061–1067.

Perry, M. C., McIntosh, M. S., Wiebold, W. J., and Welterlen, M. (1987). Genetic analysis of cold hardiness and dormancy in alfalfa. *Genome* **29**, 144–149.

Peterbauer, T., and Richter, A. (2001). Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Sci. Res.* **11**, 185–197.

Portz, H. L. (1967). Frost heaving of soil and plants. I. Incidence of frost heaving of forage plants and meteorological relationships. *Agron. J.* **59**, 341–344.

Puhakianen, T., Li, C., Boije-Malm, M., Kangasjärvi, J., Heino, P., and Palva, E. T. (2004). Short-day potentiation of low temperature-induced gene expression of a c-repeat-binding

factor-controlled gene during cold acclimation in silver birch. *Plant Physiol.* **136**, 4299–4307.

Qin, F., Sakuma, Y., Li, J., Liu, Q., Li, Y.-Q., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol.* **45**, 1042–1052.

Quarrie, S. A., Vesna, L.-J., Kovacevic, D., Steed, A., and Pekic, S. (1999). Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. *J. Exp. Bot.* **50**, 1299–1306.

Rafalski, J. A. (2002). Novel genetic mapping tools in plants: SNPs and LD-based approaches. *Plant Sci.* **162**, 329–333.

Rai, V. K. (2002). Role of amino acids in plant responses to stresses. *Biol. Plant.* **45**, 481–487.

Reaney, M. J. T., and Gusta, L. V. (1999). Modeling sequential responses of plant cells to freezing and thawing. *In* "Cold-Adapted Organisms. Ecology, Physiology, Enzymology and Molecular Biology" (R. Margesin and F. Schinner, Eds.), pp. 119–135. Springer-Verlag, Berlin.

Richmond, T., and Somerville, S. (2000). Chasing the dream: Plant EST microarrays. *Curr. Opin. Plant Biol.* **3**, 108–116.

Riday, H., and Brummer, E. C. (2002a). Forage yield heterosis in alfalfa. *Crop Sci.* **42**, 716–723.

Riday, H., and Brummer, E. C. (2002b). Heterosis of agronomic traits in alfalfa. *Crop Sci.* **42**, 1081–1087.

Riday, H., and Brummer, E. C. (2005). Heterosis in a broad range of alfalfa germplasm. *Crop Sci.* **45**, 8–17.

Rikin, A., Waldman, M., Richmond, A. E., and Dovrat, A. (1975). Hormonal regulation of morphogenesis and cold-resistance. I. Modifications by abscisic acid and by gibberellic acid in alfalfa (*Medicago sativa* L.) seedlings. *J. Exp. Bot.* **26**, 175–183.

Robertson, G. P., Paul, E. A., and Harwood, R. R. (2000). Greenhouse gases in intensive agriculture: Contributions of individual gases to the radiative forcing of the atmosphere. *Science* **289**, 1922–1925.

Rodger, J. B. A., Williams, G. G., and Davis, R. L. (1957). A rapid method for determining winterhardiness in alfalfa. *Agron. J.* **49**, 88–92.

Rohde, P., Hincha, D. K., and Heyer, A. G. (2004). Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that show differences in non-acclimated and acclimated freezing tolerance. *Plant J.* **38**, 790–799.

Roosens, N. H., Bitar, F. A., Loenders, K., Angenon, G., and Jacobs, M. (2002). Overexpression of orinthine-δ-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. *Mol. Breed.* **9**, 73–80.

Russell, W. E., Olsen, F. J., and Jones, J. H. (1978). Frost heaving in alfalfa establishment on soils with different drainage characteristics. *Agron. J.* **70**, 869–872.

Russelle, M. P. (2001). Alfalfa. *Am. Scientist* **89**, 252–261.

Sakamoto, H., Maruyama, K., Sakuma, Y., Meshi, T., Iwabuchi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). *Arabidopsis* Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiol.* **136**, 2734–2746.

Sanders, D., Brownlee, C., and Harper, J. F. (1999). Communicating with calcium. *Plant Cell* **11**, 691–706.

Sangwan, V., Övar, B. L., Beyerly, J., Hirt, H., and Dhindsa, R. S. (2002). Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct MAP kinase pathways. *Plant J.* **31**, 629–638.

Santarius, K. A., and Giersch, C. (1983). Cryopreservation of spinach chloroplast membranes by low-molecular-weight carbohydrates. *Cryobiology* **20**, 90–99.

Saravitz, D. M., Pharr, D. M., and Carter, T. E., Jr. (1987). Galactinol synthase activity and soluble sugars in developing seeds of four soybean genotypes. *Plant Physiol.* **83**, 185–189.

Schonhorst, M. H., Davis, R. L., and Carter, A. S. (1957). Response of alfalfa varieties to temperatures and daylengths. *Agron. J.* **49**, 142–143.

Schwab, P. M., Barnes, D. K., and Sheaffer, C. C. (1996). The relationship between field winter injury and fall growth score for 251 alfalfa cultivars. *Crop Sci.* **36**, 418–426.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using full-length cDNA microarrays. *Plant Cell* **13**, 61–72.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, M., *et al.* (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using full-length cDNA microarray. *Plant J.* **31**, 279–292.

Seki, M., Satou, M., Sakurai, T., Akiyama, K., Iida, K., Ishida, J., Nakajima, M., Enju, A., Narusaka, M., Fujita, M., Oono, Y., Kamei, Y., *et al.* (2004). RIKEN *Arabidopsis* full-length (RAFL) cDNA and its applications for expression profiling under abiotic stress conditions. *J. Exp. Bot.* **55**, 213–223.

Sheaffer, C. C., Barnes, D. K., Warnes, D. D., Lueschen, W. E., Ford, H. J., and Swanson, D. R. (1992). Seeding-year cutting affects winter survival and its association with fall growth score in alfalfa. *Crop Sci.* **32**, 225–231.

Shih, S. C., Jung, G. A., and Shelton, D. C. (1965). Influence of purines and pyrimidines on cold hardiness of plants. II. Respiration rate of alfalfa in relation to cold hardiness. *Crop Sci.* **5**, 307–310.

Shih, S. C., Jung, G. A., and Shelton, D. C. (1967). Effects of temperature and photoperiod on metabolic changes in alfalfa in relation to cold hardiness. *Crop Sci.* **7**, 385–389.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: Diffrences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* **3**, 217–223.

Sholar, J. R., Caddel, J. L., Stritzke, J. F., and Berberet, R. C. (1983). Fall harvest management of alfalfa in the Southern Plains. *Agron. J.* **75**, 619–622.

Silkett, V. W., Megee, C. R., and Rather, H. C. (1937). The effect of late summer and early fall cutting on crown bud formation and winterhardiness of alfalfa. *Agron. J.* **29**, 53–62.

Skøt, L., Humphreys, M. O., Armstead, I., Heywood, S., Skøt, K. P., Sanderson, R., Thomas, I. D., Chorlton, K. H., and Sackville-Hamilton, N. R. (2005). An association mapping approach to identify flowering time genes in natural populations of *Lolium perenne* (L.). *Mol. Breeding* **15**, 233–245.

Smith, D. (1951). Root branching of alfalfa varieties and strains. *Agron. J.* **43**, 573–575.

Smith, D. (1952). The survival of winter-hardened legumes encased in ice. *Agron. J.* **44**, 469–473.

Smith, D. (1961). Association of fall growth habit and winter survival in alfalfa. *Can. J. Plant Sci.* **41**, 244–251.

Smith, D. (1964). Winter injury and the survival of forage plants. *Herb. Abstr.* **34**, 203–209.

Sprague, M. A., and Fuelleman, R. F. (1941). Measurements of recovery after cutting and fall dormancy of varieties and strains of alfalfa, *Medicago sativa*. *Agron. J.* **33**, 437–447.

Sprague, M. A., and Graber, L. F. (1943). Ice sheet injury to alfalfa. *Agron. J.* **35**, 881–894.

Steponkus, P. L., and Webb, M. S. (1992). Freeze-induced dehydration and membrane destabilization in plants. *In* "Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Level" (G. N. Somero, C. B. Osmond, and C. L. Bolis, Eds.), pp. 338–362. Springer-Verlag, Berlin.

Strand, Å., Foyer, C. H., Gustafsson, P., Gardeström, O., and Hurry, V. (2003). Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies

photosynthetic acclimation at low temperatures and the development of freezing tolerance. *Plant Cell Environ.* **26**, 523–535.

Stushnoff, C., Seufferheld, M. J., and Creegan, T. (1998). Oligosaccharides as endogeneous cryo-protectants in woody plants. *In* "Plant Cold Hardiness: Molecular Biology, Biochemistry and Physiology" (P. H. Li and T. H. H. Chen, Eds.), pp. 301–309. Plenum Press, New York.

Sulc, R. M., Albrecht, K. A., and Duke, S. H. (1991a). Leakage of intracellular substances as an indicator of freezing injury in alfalfa. *Crop Sci.* **31**, 430–435.

Sulc, R. M., Albrecht, K. A., Palta, J. P., and Duke, S. H. (1991b). Leakage of intracellular substances from alfalfa roots at various subfreezing temperatures. *Crop Sci.* **31**, 1575–1578.

Summaryati, S., Negrutiu, I., and Jacobs, M. (1992). Characterization and regeneration of salt- and water-stress mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani). *Theor. Appl. Genet.* **83**, 613–619.

Svenning, M. M., Røsnes, K., and Junttila, O. (1997). Frost tolerance and biochemical changes during hardening and dehardening in contrasting white clover populations. *Physiol. Plant.* **101**, 31–37.

Swanson, C. R., and Adams, M. W. (1959). Metabolic changes induced in alfalfa during cold hardening and freezing. *Agron. J.* **51**, 397–400.

Taji, T., Ohsumi, C., Luchi, S., Seki, M., Kasuga, M., Kobayashi, M., Ymaguchi-Shinozaki, K., and Shinozaki, K. (2002). Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.* **29**, 417–426.

Tesar, M. B., and Yager, J. L. (1985). Fall cutting of alfalfa in the North Central USA. *Agron. J.* **77**, 774–778.

Teuber, L. R., Taggard, K. L., Gibbs, L. K., McCaslin, M. H., Peterson, M. A., and Barnes, D. K. (1998). Fall dormancy. *In* "Standard Tests to Characterize Alfalfa Cultivars," 3rd Ed., p. A-1 [online]. Available at http://www.naaic.org/stdtests/Dormancy2.html (accessed 26 May 2006; verified 26 May 2006). North Amer. Alfalfa Improvement Conf., Beltsville, MD.

Theurer, J. C., and Elling, L. J. (1963). Comparative performance of diallel crosses and related second generation synthetics of alfalfa. II. Winter hardiness and persistence. *Crop Sci.* **3**, 245–248.

Thomashow, M. F. (1998). Role of cold responsive genes in plant freezing tolerance. *Plant Physiol.* **118**, 1–7.

Thomashow, M. F. (2001). So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* **125**, 89–93.

Timmons, F. L., and Salmon, S. C. (1932). The resistance of certain varieties and regional strains of alfalfa to controlled low temperatures. *Agron. J.* **24**, 642–655.

Uemura, M., Joseph, R. A., and Steponkus, P. L. (1995). Cold acclimation of *Arabidopsis thaliana*. Effect on plasma membrane lipid composition and freeze-induced lesions. *Plant Physiol.* **109**, 15–30.

Ukaji, N., Kuwabara, C., Arakawa, K., and Fujikawa, S. (2004). Accumulation of pathogenesis-related (PR) 10/Bet v 1 protein homologues in mulberry (*Morus bombycis* Koidz.) tree during winter. *Plant Cell Environ.* **27**, 1112–1121.

Van Keuren, R. W. (1988). Frost heave of alfalfa as affected by harvest schedule. *Agron. J.* **80**, 626–631.

Vision, T. J. (2005). Gene order in plants: A slow but sure shuffle. *New Phytol.* **168**, 51–60.

Vogel, J. T., Zarka, D. G., Van Buskirk, H. A., Fowler, S. G., and Thomashow, M. F. (2005). Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J.* **41**, 195–211.

Volenec, J. J., Cunningham, S. M., Haagenson, D. M., Berg, W. K., Joern, B. C., and Wiersma, D. W. (2002). Physiological genetics of alfalfa improvement: Past failures, future prospects. *Field Crop Res.* **75**, 97–110.

Wang, L. C., Attoe, O. J., and Truog, E. (1953). Effect of lime and fertility levels on the chemical composition and winter survival of alfalfa. *Agron. J.* **45**, 381–384.

Wang, Y. J., Zhang, Z.-G., He, X.-J., Zhou, H.-L., Wen, Y.-X., Dai, J. X., Zhang, J. S., and Chen, S.-Y. (2003). A rice transcription factor OsbHLH1 is involved in cold stress response. *Theor. Appl. Genet.* **107**, 1402–1409.

Wanner, L. A., and Junttila, O. (1999). Cold-induced freezing tolerance in *Arabidopsis*. *Plant Physiol.* **120**, 391–399.

Weiser, C. J. (1970). Cold resistance and injury in woody plants. *Science* **69**, 1269–1278.

Weishaar, M. A., Brummer, E. C., Volenec, J. J., Moore, K. J., and Cunningham, S. M. (2005). Improving winterhardiness in nondormant alfalfa germplasm. *Crop Sci.* **45**, 60–65.

Wiersma, D. W., Undersander, D. J., and Grau, C. R. (1997). Root heave of alfalfa cultivars with differing levels of resistance to aphanomyces root rot. *Agron. J.* **89**, 148–150.

Willard, C. J. (1930). Root reserves of alfalfa with special reference to time of cutting and yield. *Agron. J.* **22**, 595–602.

Willekens, H., Chammnongpol, S., Davey, M., Schraudner, M., Langerbartels, C., Van Montagu, M., Inze, D., and Van Camp, W. (1997). Catalse is a sink for H_2O_2 and is indispensable for stress defence in C-3 plants. *EMBO J.* **16**, 4806–4816.

Wise, M. J. (2003). LEAping to conclusions: A computational reanalysis of late embryogenesis abundant proteins and their possible roles. *BMC Bioinformatics* **4**, 52. Available at: http://www.biomedcentral.com/1471-2195/4/52.

Wisniewski, M. E., Webb, R., Balsamo, R., Close, T. J., Xu, X.-M., and Griffith, M. (1999). Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: A dehydrin from peach (*Prunus persica*). *Physiol. Plant.* **105**, 600–608.

Wolfraim, L. A., and Dhindsa, R. S. (1993). Cloning and sequencing of the cDNA for *cas*17, a cold acclimation-specific gene of alfalfa. *Plant Physiol.* **103**, 667–668.

Wolfraim, L. A., Langis, R., Tyson, H., and Dhindsa, R. S. (1993). cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas*18, of alfalfa (*Medicago falcata*) cells. *Plant Physiol.* **101**, 1275–1282.

Wolkers, W. F., McCready, S., Brandt, W. F., Lindsey, G. G., and Hoekstra, F. A. (2001). Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses *in vitro*. *Biochem. Biophys. Acta* **1544**, 196–206.

Wong, C. E., Li, Y., Whitty, B. R., Díaz-Camino, C., Akhter, S. R., Brandle, J. E., Golding, G. B., Weretilnyk, E. A., Moffatt, B. A., and Griffith, M. (2005). Expressed sequence tags from the Yukon ecotype *Thellungiella* reveal the gene expression in response to cold, drought and salinity shows little overlap. *Plant Mol. Biol.* **58**, 561–574.

Woolley, D. G., and Wilsie, C. P. (1961). Cold unit accumulation and cold hardiness of alfalfa. *Crop Sci.* **1**, 165–167.

Xin, Z., and Browse, J. (2000). Cold comfort farm: The acclimation of plants to freezing temperatures. *Plant Cell Environ.* **23**, 893–902.

Xiong, L., Ishitani, M., Lee, H., and Zhu, J.-K. (2001). The *Arabidopsis* LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic-stress-responsive gene expression. *Plant Cell* **13**, 2063–2083.

Yan, J., Wang, J., Tissue, D., Holaday, A. S., Allen, R., and Zhang, H. (2003). Photosynthesis and seed production under water-deficit conditions in transgenic tobacco plants that over-express an *Arabidopsis* ascorbate peroxidase gene. *Crop Sci.* **43**, 1477–1483.

Ye, B., Muller, H. H., Zhang, J., and Gressel, J. (1997). Constitutively elevated levels of putrescine and putrescine-generating enzymes correlated with oxidant stress resistance in *Conyza bonariensis* and wheat. *Plant Physiol.* **115**, 1443–1451.

Yeh, S., Moffatt, B. A., Griffith, M., Xiong, F., Yang, D. S. C., Wiseman, S. B., Sarhan, F., Danyluk, J., Xue, Y. Q., Hew, C. L., Doherty-Kirby, A., and Lajoie, G. (2000). Chitinase

genes responsive to cold encode antifreeze proteins in winter cereals. *Plant Physiol.* **124**, 1251–1263.

Yi, S. Y., Kim, J.-H., Joung, Y.-H., Lee, S., Kim, W. T., Yu, S. H., and Choi, D. (2004). The pepper transcription factor *CaPF1* confers pathogen and freezing tolerance in *Arabidopsis*. *Plant Physiol.* **136**, 2862–2874.

Yun, D.-J., Bressan, R. A., and Hassegawa, P. M. (1997). Plant antifungal proteins. *Plant Breed. Rev.* **14**, 39–88.

Zarka, D. G., Vogel, J. T., Cook, D., and Thomashow, M. F. (2003). Cold-induction of Arabidopsis *CBF* genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol.* **133**, 910–918.

Zhang, J. Z., Creelman, R. A., and Zhu, J.-K. (2004a). From laboratory to the field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol.* **135**, 615–621.

Zhang, X., Fowler, S. G., Cheng, H., Lou, Y., Rhee, S. Y., Stockinger, E. J., and Thomashow, M. F. (2004b). Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J.* **39**, 905–919.

Zhang, J.-Y., Broeckling, C. D., Blancaflor, E. B., Sledge, M. K., Sumner, L. W., and Wang, Z.-Y. (2005). Overexpression of *WXP1*, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* **42**, 689–707.

Zhu, H., Choi, H.-K., Cook, D. R., and Shoemaker, R. C. (2005a). Bridging model and crop legumes through comparative genomics. *Plant Physiol.* **137**, 1189–1196.

Zhu, J., Shi, H., Lee, B.-H., Dammsz, B., Cheng, S., Stirm, V., Zhu, J.-K., Hasegawa, P. M., and Bressan, R. A. (2004). An *Arabidopsis* homeodomain transcription factor gene, *HOS9*, mediates cold tolerance through a CBF-independent pathway. *Proc. Natl. Acad. Sci.* **101**, 9873–9878.

Zhu, J., Verslues, P. E., Zheng, X., Lee, B. H., Zhan, X., Manabe, Y., Sokolchik, I., Zhu, Y., Dong, C. H., Zhu, J.-K., Hasegawa, P. M., and Bressan, R. A. (2005b). *HOS10* encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. *Proc. Natl. Acad. Sci.* **102**, 9966–9971.

Zhu, T., and Provart, N. J. (2003). Transcriptional responses to low temperature and their regulation in. *Arabidopsis. Can. J. Bot.* **81**, 1168–1174.

Zúñiga-Feest, A., Ort, D. R., Gutiérrez, A., Gidekel, M., Bravo, L., and Corcuera, L. J. (2005). Light regulation of sucrose-phosphate synthase activity in the freezing tolerant grass *Deschampsia antartica. Photosynth. Res.* **83**, 75–86.

This page intentionally left blank

Projecting Yield and Utilization Potential of Switchgrass as an Energy Crop

Samuel B. McLaughlin,[1] James R. Kiniry,[2]
Charles M. Taliaferro[3] and Daniel De La Torre Ugarte[4]

[1]Bioenergy Feedstock Development Program, Environmental Sciences Division,
Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831
[2]USDA Agricultural Research Service, Grassland,
Soil and Water Research Laboratory, Temple, Texas 76502
[3]Department of Plant and Soil Sciences,
Oklahoma State University, Stillwater, Oklahoma 74078
[4]US Department of Energy, Agricultural Policy Analysis Center,
University of Tennessee, Knoxville, Tennessee 37996

The potential utilization of switchgrass (*Panicum virgatum* L.) as a cellulosic energy crop was evaluated as a component of a projected future national network of biorefineries designed to increase national reliance on renewable energy from American farms. Empirical data on yields of switchgrass from a network of experimental plots were coupled with data on switchgrass physiology and switchgrass breeding progress to provide reasonable expectations for rates of improvement over current yields. Historical

breeding success with maize (*Zea mays* L.) was found to provide a reasonable model for projected linear rates of yield improvement of switchgrass based on documented progress to date. A physiologically based crop production model, ALMANAC, and an econometric model, POLYSYS, were utilized to estimate variability in switchgrass yield and resource utilization across the eastern two-thirds of the United States. ALMANAC provided yield estimates across 27 regional soil types and 13 years of weather data to estimate variability in relative rates of production and water use between switchgrass and maize. Current and future yield projections were used with POLYSYS to forecast rates of adaptation and economic impacts on regional agricultural markets. Significant positive impacts on US markets, including significant increases in farm income and significant reduction in the need for government subsidies, were projected. This was based on expected technological progress in developing biorefineries that will significantly increase national energy self-sufficiency by producing feed protein, transportation fuel, and electrical power from cellulosic feedstocks.   © 2006, Elsevier Inc.

I.   INTRODUCTION

While ethanol from maize is the dominant means by which renewable energy is channeled from sunlight to the transportation industry (Shapouri *et al.*, 1995), switchgrass has become another strong candidate for production of bioenergy. Switchgrass is a native perennial, warm-season grass species within which selection has been practiced for forage and conservation uses over the past half-century (Vogel *et al.*, 1985). In 1991 it was selected as a candidate for utilization in production of bioenergy and bioproducts (McLaughlin and Kszos, 2005). Its strongest attributes include high biomass production capability and energy recovery capacity with low energy and material inputs, and excellent compatibility with existing agricultural practices. These qualities, combined with strong soil and water conservation values, and a high capacity to reduce emissions of greenhouse gases have led to switchgrass being considered as a potentially important component of a national energy strategy (McLaughlin *et al.*, 2002).

Despite criticism of ethanol production from maize based on low energy efficiency and adverse environmental impacts (Pimmentel *et al.*, 2002), maize-based ethanol production has made an important beginning in the reduction of reliance of the United States on imported oil. Maize-based ethanol does displace significantly more oil than is used in its production (Shapouri *et al.*, 1995). However, McLaughlin and Walsh (1998) suggested that the efficiency of energy conversion and reduction of greenhouse emissions through production of cellulosic ethanol from switchgrass could

exceed that from maize ethanol by more than an order of magnitude. Yet maize remains the standard biofuel feedstock, which provides a base that can ultimately be supplemented by other feedstocks, providing greater economic and environmental efficiencies.

If switchgrass is to provide a viable supplement to ethanol from maize, biomass production levels of switchgrass must be determined as input for a national renewable energy strategy. The Role of Biomass in America's Energy Future (RBAEF) project was initiated to help formulate such a strategy. The RBAEF project represents the most comprehensive effort in the United States to date that has focused on analysis of mature technology for production of energy from biomass. It has involved experts in bioenergy analysis from government and university coupled with active involvement of both conservation (Natural Resources Defense Council) and policy (Office of Energy Policy) organizations. The RBAEF project has considered over 20 mature process technology scenarios for production of a broad range of fuels and electrical power from cellosic biomass. Reasonably optimistic forecasts for both biomass production and bioenergy conversion were evaluated for a projected national network of biorefineries that could contribute to national energy self-sufficiency (Greene, 2004). Switchgrass was selected as the model crop for this study. In that context the research described herein has formed a basis for considering what role switchgrass could play in a national energy supply system. Such a system would incorporate the best foreseeable technology to produce energy and value-added products such as animal feed protein from cellulosic feedstocks. Yield levels will play a key role in the economics of such production and utilization systems as well as in determining the demographics of production.

When discussing methods for increasing plant biomass yield, some terms describing yield must be defined. Two such terms commonly used are "yield potential" and "potential yield." As used in this study, yield potential is the maximum yield (biomass or grain) levels that have been attained at any time for a specific genotype of a crop or grass under field conditions. In contrast, potential yield is the maximum predicted yield based on simulations founded in plausible physics, biochemistry, and physiology of the crop in its normal growing environment (Fischer and Evans, 1999). This yield is considered theoretically and physiologically possible based on maximum light interception and biochemical conversion of solar radiation into dry matter accumulation.

Because maize production is a cornerstone of agricultural economics in North America, the historical improvement of maize yields represents an important standard from which to project future yield gains of other species with comparable production characteristics. Maize yield records for North America extend back more than 100 years and provide a template for both defining and understanding yield improvement through breeding and crop

physiological studies (Duvick, 1997; Tollenaar *et al.*, 1994). Maize and switchgrass not only share the common trait of being useful bioenergy crops but are also similar in that both are warm-season, C_4 species. However, maize is an annual with only the grain used for ethanol production while switchgrass is a perennial with the entire aboveground biomass used when energy is the endpoint. Maize is a good standard of comparison because of the extensive breeding for increased yields and the extensive physiological research on processes contributing to yield. Investigation of the physiology and breeding history of these two plant species, as related to increased yields, becomes important for studies of yield potential as a theoretical upper limit of yield increases achievable through breeding.

In this chapter, we examine the past record of yield improvement in maize and the basis of those gains to provide a framework for projecting gains in yield of switchgrass. A necessary component of these analyses has been comparisons of the agronomic characteristics, breeding history, and underlying physiology of maize and switchgrass. We had three objectives in initiating this study. First, we wanted to evaluate potential yield improvement in switchgrass using maize breeding advances as a model. Second, we wanted to test and apply a physiologically based crop production model, ALMANAC (Kiniry *et al.*, 1992), parametrized to switchgrass physiology to estimate both potential yield and yield potential of switchgrass. Finally, we wanted to describe links between productivity and production costs for regional projections of switchgrass utilization that would require widespread participation of the agricultural community of the United States in supporting renewable energy production. Such participation must be based on switchgrass providing attractive economic alternatives to conventional crops. For these analyses we have used the econometric model POLYSYS (Ugarte and Ray, 2000).

II. PROJECTING YIELD GAINS IN SWITCHGRASS RELATIVE TO MAIZE

A. BREEDING HISTORY OF MAIZE

While maize was domesticated more than 7000 years ago (Goodman and Brown, 1988), the largest increases in yields occurred in the past 75 years as modern breeding techniques evolved (Duvick, 1997, 1999; Tollenaar *et al.*, 1994). Switchgrass breeding has a much shorter history, with selection for yield increases and trait improvement having occurred only in the last few decades. However, this genetically diverse grass is an important component

of the North American tallgrass prairies and has undergone thousands of years of natural selection for persistence under the stresses imposed by grazing animals, principally bison (*Bison bison*), and fires (Steinauer and Collins, 1996).

Since 1930, maize yield increases in the United States and Canada have been remarkably linear (Fig. 1) (derived from http://www.nass.usda.gov/Charts_and_Maps/Field_Crops/cornyld.asp). The record of breeding progress in maize extends back over 130 years (Tollenaar *et al.*, 1994) and reflects definitive stages of progress during the evolution of breeding approaches. Breeding strategies have progressed from initial mass selection in open-pollinated populations to the breeding technology capitalizing on hybrid vigor captured in single crosses, double crosses, and finally, three-way crosses. Breeding for hybrid vigor began in maize in the early 1900s. The introduction of the first double cross hybrids in the 1930s and 1940s provided the largest gains in yields. Yields in the late 1990s achieved levels nearly five times those in the early 1930s. The mean rate of gain was 80–100 kg ha^{-1} year^{-1} for the United States. It is generally considered that, while heterosis has played an important role in maize hybrid yield increases and will continue to contribute to yield gains, the importance of heterosis to absolute gains will likely decrease as inbreeding raises baseline yields (Duvick, 1999).

The impressive maize yield gains have come only in part due to genetic improvements. Genetic gains have contributed 50–60% to the overall yield gains achieved with the remainder due to improved management (Duvick, 1999). Gains in maize yield potential have been accompanied by improved physiological characteristics, making plants more resistant to stresses inherent at high-planting density. Understanding roles such changes have played
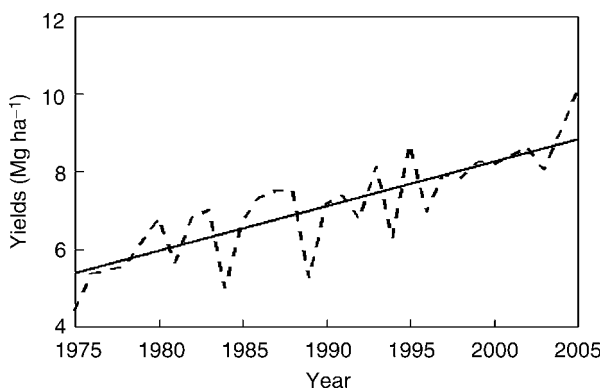
Figure 1   US maize yield gains in the last 30 years. US Department of Agriculture.

in yield gains is important to our objective of estimating how such gains can be mirrored with switchgrass.

B. BREEDING GAINS WITH PERENNIAL GRASSES INCLUDING SWITCHGRASS

Scientific breeding of perennial grasses largely began in the mid-20th century and has been on a much smaller scale than for maize. Consequently, performance gains attributable to sustained breeding of perennial grasses, in general, are much less than for maize. Additionally, the number, scope, and duration of breeding programs have varied greatly with perennial grass species, contributing to substantial differences among species in breeding gains.

Articles addressing genetic gains, made mainly in important cool-season perennial grass species, indicate differences associated with traits, species, and geographic regions (Casler, 2001; Casler *et al.*, 2000; Wilkins and Humphreys, 2003). Casler *et al.* (2000) compared smooth bromegrass (*Bromus inermis* Leyss.) cultivars developed in the United States between 1942 and 1995 with cultivars predating 1942. While no genetic gains in dry biomass yields (DBY) were detected among the cultivars developed between 1942 and 1995, the DBY of the post 1942 cultivars averaged 0.54 Mg ha^{-1} year^{-1}. Their mean DBY was 7.2% greater than the DBY of ''Lincoln,'' a direct representative of the first smooth bromegrass introduced into North America. They also reported small gains in brown leaf-spot resistance (0.21 units per decade), *in vitro* dry matter digestibility (IVDMD) (9 g kg^{-1}, 1.4%), and neutral detergent fiber (NDF) (–8 g kg^{-1}, −1.2%). The slow rate of genetic gain for DBY was attributed to the complex polyploid inheritance of smooth bromegrass, breeding emphasis on traits other than forage yield, and relatively little concerted attention from public and private breeders. Casler (2001) reviewed breeding efforts for improved forage nutritional value, reporting enhancements for several different species and indices (increased IVDMD, nylon bag dry matter digestibility, and protein, and decreased acid detergent fiber and lignin) ranging from 1 to 7% per cycle. Wilkins and Humphreys (2003) reported that over the past 50 years, gains in DBY of the important forage grass species have been 4–5% per decade in northwestern Europe, but only 0–1% per decade in the United States. Additionally, they state that gains in the dry matter digestibility of perennial ryegrass (*Lolium perenne* L.) in the United Kingdom have been 10 g kg^{-1} per decade, whereas in the United States little or no gain has been made, presumably reflecting the continental differences in amount of breeding effort and/or breeding objectives. Common expressions in the previously cited articles are that genetic gains in the perennial grasses have been limited mainly by lack of breeding

effort and that the largely unmined genetic diversity within these species offers potential for enormous breeding improvement.

Significant breeding advances have been documented in several warm-season (C_4) perennial grasses for DBY and other attributes. One well-known success story is with bermudagrass [*Cynodon dactylon* (L.) Pers.]. Bermudagrass breeding initiated by Burton in 1937 at Tifton, GA led to the release of "Coastal" bermudagrass in 1943 (Burton, 1947, 1954). Coastal bermudagrass DBY is nearly twice that of unselected common bermudagrass strains found in the southeastern United States (Adams and Stelly, 1958; Carreker *et al.*, 1972). In addition, Burton (1982, 1985, 1992) and Burton and Mullinix (1998) increased DBY of bahiagrass (*Paspalum notatum* Flügge var *saurae* Parodi) through systematic restricted recurrent phenotypic selection (RRPS). Mean individual spaced plant DBY increased in bahiagrass population "A" from 364 g per plant in cycle 0 to 1767 g per plant in cycle 18, a gain in mean individual plant DBY of 78 g per RRPS cycle. In narrow base population "B," mean spaced plant DBY increased from 823 g per plant in cycle 0 to 1427 g per plant in cycle 10, a gain in mean individual plant DBY of 60 g per RRPS cycle. While RRPS increased the number of high-yielding plants and reduced the number of low-yielding plants in successive cycles, genetic variation for DBY remained high in the populations. Population A RRPS cycle 18 had two plants with DBY of 4540 g and 27 plants with DBY of only 454 g.

Systematic breeding within switchgrass populations specifically for increased DBY is in its infancy, and yield gains of the first few breeding cycles have just been reported (McLaughlin and Kszoz, 2005). Many commonly grown switchgrass cultivars are direct increases of naturally occurring strains (Vogel, 2000, 2004). Examples include the lowland ecotypes "Alamo" and "Kanlow," and the upland ecotypes "Blackwell" and "Cave-in-rock." Cyclic selection in switchgrass for increased nutritive value has been effective and has resulted in the release of "Trailblazer" and "Shawnee," upland type cultivars adapted to the central United States (Vogel, 2004).

The potential for increasing DBY in switchgrass is significant because of the large genetic variation within the species. There is substantial heritable variation in switchgrass for DBY and related performance traits (Hopkins *et al.*, 1993; Newell and Eberhart, 1961; Talbert *et al.*, 1983; Taliaferro *et al.*, 1999; Vogel *et al.*, 1981). Additional basic information generated over the past decade has strengthened understanding of the biology of the species, providing a firm foundation for applied breeding improvement.

Future breeding gains in switchgrass DBY will depend on the amount and consistency of effort expended and on continued refinement of breeding protocols. Better screens are needed to enhance the effectiveness and efficiency of selection. The RRPS protocol, used so successfully with bahiagrass, did not achieve the desired results with switchgrass in Nebraska and Oklahoma

(Hopkins *et al*., 1993; Taliaferro, 2002). Two cycles of RRPS failed to increase DBY in an upland population, but did provide linear gains in IVDMD through three cycles (Hopkins *et al*., 1993). Three cycles of RRPS for increased DBY in each of four populations (two upland and two lowland) gave generally desirable but mixed results in Oklahoma (Taliaferro, 2002). Yield gains per cycle varied from near 0 to a maximum of 6% and were not linear across cycles. Use of RRPS was relatively ineffective in identifying plants of superior breeding value in the establishment year, in large part because much of the growth during that year is belowground and not readily assessed by these techniques. Some switchgrass breeding programs are now using progeny testing as a basis for selection of plants with superior breeding value for DBY, a process known as genotypic recurrent selection. This procedure has been successful with many crops for many traits but is the most time consuming of the recurrent selection procedures (Fehr, 1987). Four to five years are required for each cycle.

Rapid progress has been made using a novel honeycomb planting design (Fasoula and Fasoula, 1995) to promote phenotypic selection within a lowland switchgrass population developed from Alamo and Kanlow (Bouton, 2002). Data from preliminary testing indicated four synthetic cultivars developed using elite plants selected from the population had 30% yield gains relative to parent populations of Alamo and Kanlow. The selection process occurred over 4 years and equated to an annual gain of 7.5%. This compares favorably with early gains in maize improvement in the United States, which ranged from 3.5–6.0% of baseline yields in the 1930s to 1.3–1.8% in the 1990s (Tollenaar *et al*., 1994). Average yield gains for maize made over 70 years of breeding for commercial markets in Iowa have, as expected, been lower (0.7–1.2% per year) (Duvick, 1997).

C. POTENTIAL YIELDS OF MAIZE AND SWITCHGRASS

Our analyses suggest that upper limit yields (potential yields) of maize and switchgrass are similar. In simplest terms, potential yield is governed by how much radiant energy can be captured by the plant canopy and converted to harvestable biomass over an annual growing season. The key processes regulating potential yield are the leaf canopy size and longevity, and efficiency of canopy interception of radiant energy; the efficiency of conversion of radiant energy to photosynthetic products and plant biomass; and the relative allocation of carbohydrates to the physical and metabolic support of the whole plant system. Loomis and Amthor (1996, 1999) concluded that genetic control of photosynthesis and respiration is complex and relatively stable, such that the basic efficiency of these processes appears little

altered by crop domestication and breeding. They estimated that radiation use efficiency (RUE) of maize should be 4.6 g MJ^{-1} of intercepted photosynthetically active radiation. However, measured RUE values with high-yielding maize in the field are only 3.7–3.8 (Kiniry *et al.*, 2004; Lindquist *et al.*, 2005; Tollenaar and Aguilar, 1992).

While neither the upper limits of photosynthesis nor the upper limits of yield potential have changed measurably (Tollenaar and Wu, 1999), the yielding ability of maize in the field has increased dramatically (Fig. 1). This is because maize yields are affected by tolerances to natural stress and responsiveness to management inputs (Fasoula and Fasoula, 2000).

A side benefit of selecting higher maize yield has been increased resistance to stresses associated with high-yield production systems (Duvick, 1997; Tollenaar and Lee, 2002; Tollenaar and Wu, 1999; Tollenaar *et al.*, 1994). Among the performance traits that have improved as maize yields have increased are: increased resistance to competition in high-density plantings (Tokatlidis, 2001); increased resistance to drought stress (Dwyer *et al.*, 1992); improved nitrogen use efficiency (McKay and Barber, 1986); reduced dark respiration (Earl and Tollenaar, 1998); improved source:sink relations (Rajcan and Tollenaar, 1999); improved canopy level efficiency at interception and utilization of radiation (Dwyer *et al.*, 1991); and increased longevity of the productive maize canopy, a phenomenon referred to as ''stay-green'' (Tollenaar *et al.*, 1994). While these traits have not played significant roles in breeding strategies to date, they could be important for increased future yields of maize or switchgrass. Their improvement for maize has been attributed to the trend in commercial breeding to include testing and selection for performance across diverse testing environments, which included wide variations in these stresses (Tollenaar and Lee, 2002).

Maximum grain yield potential attained by maize under field conditions is in the range of 15–20 Mg ha^{-1} (Tollenaar and Wu, 1999). These data represent record yields from a few individual fields. Maximum regional yields and countrywide commercial yields are lower than these maxima, in the range of 11 Mg ha^{-1} for individual counties (National Agricultural Statistical Service, ftp://www.nass.usda.gov/pub/county/byyear/) to 13 Mg ha^{-1} (5-year average) for the highest yielding hybrids in Texas (Pietsch *et al.*, 1999). Yield potential of such irrigated maize in the field is 36–48% below potential yield, which has a theoretical maximum of 25 Mg ha^{-1} (Tollenaar, 1983). Average commercial yields of maize between 1980 and 2000 in central North America were considerably less, in the range of 6–7 Mg ha^{-1} (Tollenaar and Lee, 2002).

Yields of switchgrass have been evaluated in the Mid-Atlantic, Southeast, South-Central, and Northern Plains states through a network of field plots designed to evaluate existing commercially available varieties as well as new cultivars developed in an emerging breeding program (Fig. 2). These plots
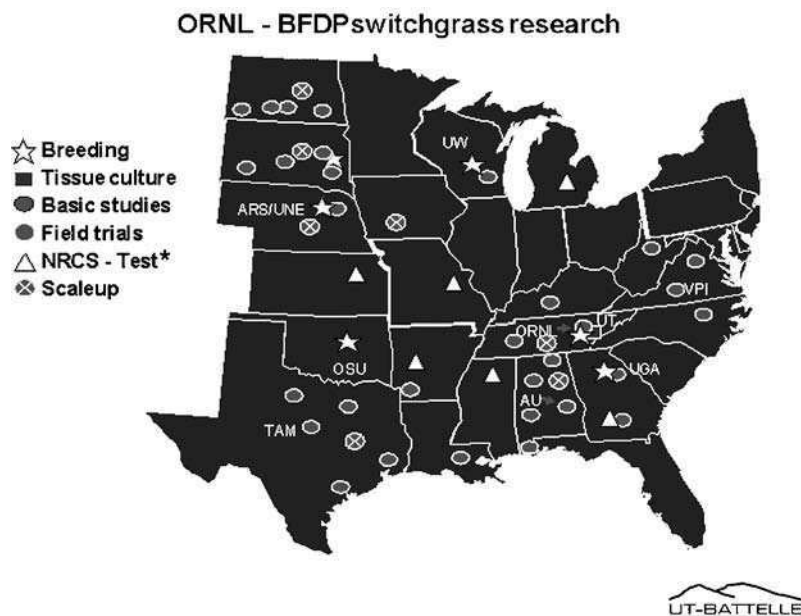
Figure 2 Distribution of yield test sites and basic research activities supporting yield estimates and yield improvement potential in this study (after McLaughlin *et al.*, 1999). The Oak Ridge National Laboratory (ORNL) Bioenergy Feedstock Development Program (BFDP) was a 10-year research program sponsored by the Department of Energy. *USDA-NRCS Plant Materials Centers–variety evaluation.

have served to identify both the most productive varieties for various latitudes and to evaluate the influences of various management regimes, including cutting regimes (one or two harvests per year with variable harvesting dates), nitrogen form and application rates, and row spacing (45–120 cm). Early yield gains of 50% were made by identifying varieties that were best suited to each production region (McLaughlin and Kszos, 2005).

The best available varieties identified over the 1991–2000 test period were the lowland ecotypes Alamo and Kanlow in the southern latitudes; Kanlow and an upland ecotype Cave-in-rock at mid-latitudes; and Cave-in-rock and another upland variety, Summer, in the northern latitudes. Average annual yields of the best adapted varieties in each region over 5–8 years (Table I) have ranged from 11 to 23 Mg ha^{-1} with a maximum individual plot/variety yield of 35 Mg ha^{-1} year^{-1} in Alabama (Sladden *et al.*, 1991). These yields have been produced without irrigation on sites selected to represent agricultural land of moderate quality that would not likely be used for dominant cash crops such as maize or soybeans [*Glycine max* (L.) Merr.].

Table I
Annual Switchgrass Productivity in a Variety of US Test Environments in the Field

Location	Sites	Time (years)	Yield (Mg ha^{-1} year^{-1}) Average	Range	Maximum site
Mid-Atlantic	8	8	13.9	10.9–17.5	27.4
Georgia	2	5	16.2	16.1–16.3	23.2
Alabama	1	13	23.0	NA	34.6
Alabama	5	8	12.9	10.4–15.8	24.6
Texas	3	6	13.5	8.1–16.5	24.7
Iowa	1	4	13.1	NA	17.5
Nebraska	1	3	20.6	NA	—
North Dakota	2	2	11.0	9.8–12.2	13.8

Average, range, and maximum 1-year yields of the best switchgrass varieties by site were determined by standard agricultural test plots.

D.   Whole Plant Production in Maize and Switchgrass

Because harvested maize grain crop represents allocation of a portion of the captured and converted solar energy from the maize canopy, one must also know the harvest index (grain weight divided by total aboveground plant weight) to calculate total productivity of the maize crop. This is particularly relevant in comparing results of yield increases of a grain crop, like maize, with a cellulosic crop, like switchgrass, where total biomass production is the major emphasis. The harvest index of maize is 50–54% (Echarte and Andrade, 2003; Kiniry et al., 2002, 2004; Tollenaar, 1992). Unlike the increasing harvest index values that have tracked the trend in yield gains in some of the cereal grains, such as wheat (Fischer et al., 1998), the harvest index of maize has remained largely unchanged (Tollenaar, 1989). With a maximum theoretical yield for maize of 25 Mg ha^{-1} and a harvest index of 54% (Echarte and Andrade, 2003; Kiniry et al., 2004), the calculated maximum aboveground productivity of maize is 46.3 Mg ha^{-1}. Potential yield of maize on an individual plant basis is considered to have remained largely unchanged over the last several decades of yield increases (Duvick, 1997). What has improved is the performance of leaf canopies under intensive management for high yields.

Estimates of maximum potential yields of switchgrass are similar to those of maize. Initial estimates of potential yields of switchgrass came from a space-planted nursery in eastern Tennessee at a location with the highest overall yields attained during the yield trials (Table II). Individual plants grown on a 1.2 m × 1.2 m spacing were harvested and weighed to determine size distribution within a 1000-plant nursery initiated from seedlings produced in a greenhouse from tissue culture explants (Conger et al., 1996).

Table II
Some Physiologically Based Production Characteristics of Switchgrass Based on Field Measurements of Accessions at a Germplasm Nursery in Tennessee, Oklahoma, and Texas (see text)

Knoxville, Tennessee
Maximum single-plant yield in a space-planted nursery from a 1000-plant nursery in tennessee (projected from 1.2 m × 1.2 m spaced planting)

Average yield all plants:		20.6 Mg ha^{-1} year^{-1}
Most frequent yield:		22.9 Mg ha^{-1} year^{-1}
Highest yielding plant:		7.0 kg
"Max plot"[a]:		48.6 Mg ha^{-1} year^{-1}

Stillwater, Oklahoma 1999
Physiological characteristics

Single-leaf photosynthesis	Range:	17.5–30.8 µmol m^{-2} s^{-1}
	Top 3:	30.5
	Alamo:	27.9
Transpiration	Range:	6.2–13.0 mmol m^{-2} s^{-1}
	Top 3:	11.83
	Alamo:	8.2
Stomatal conductance	Range:	0.16–0.30 mol m^{-2} s^{-1}
	Top 3:	0.29
	Alamo:	0.23
Water use efficiency	Range:	2.08–3.77 µmol mmol^{-1}
(Ps/Tr)	Top 3(WUE):	3.71
	Top 3(Ps):	2.67
	Alamo:	3.6
Dark respiration (4 varieties at 3 sites)	Range:	1.76–2.24 µmol m^{-2} s^{-1}
	3 Site average:	2.12
	Alamo:	1.98

Stevenville, Texas 1993

Seasonal Ps	Alamo:	
	Early (May 18):	30.6 µmol m^{-2} s^{-1}
	Later (July 16):	18.1
	Cave-in-rock	
	Early (May 18):	27
	Later (July 16):	16.2

[a]This is a projection of the yield potential of a field of plants all of which have the productive potential of the best plant in this 0.40 ha unirrigated test plot with a 1.2 m × 1.2 m planting density.
WUE, water use efficiency; Ps, photosynthesis; Tr, transpiration.
Source: Wullschleger et al. (1996a,b).

These plots produced some of the highest yields in the test network in 1995. Estimates of the upper limits of field-scale yields based on the distribution of yields within the stand projected a maximum of 48.6 Mg ha^{-1} year^{-1} (based on a population at the level of the highest yielding plant). Similar calculations based on the mode and average yields of individual plants were 22.9 and 20.6 Mg ha^{-1} year^{-1}, respectively.

The estimates of maximum annual potential yields of maize (46.3 Mg ha^{-1} year^{-1}) and switchgrass biomass (48.6 Mg ha^{-1} year^{-1}) (Table II) are remarkably similar, despite the differences in life strategy. Although both have the efficient C_4 metabolism, the perennial life strategy of switchgrass dictates that the species' persistence rests strongly on energy storage and mobilization from a much larger root system than the annual crop maize, which invests half of annual dry matter accumulation in grain. Estimates of the root mass of switchgrass from excavation studies at eight sites in the mid-Atlantic region averaged 15.1 Mg ha^{-1} and ranged from 31% to 60% of the total biomass above and below ground at harvest (Parrish et al., 2003). Similar work with Alamo switchgrass roots sampled with soil coring at final harvest in Texas showed that 30% and 37% of the total biomass were in the roots in a wet year and 60% and 73% in a dry year (Kiniry et al., 1999).

III. PROJECTING SWITCHGRASS PERFORMANCE IN TIME AND SPACE WITH THE ALMANAC MODEL

A. Physiological and Ecological Traits of Switchgrass

While physiological criteria have had limited utility in increasing maize yields, the effects of maize breeding for greater yield on physiological traits indicate that such characteristics may be valuable in targeting increased yield in future breeding efforts when applied with the tools of molecular biology (Tollenaar et al., 1994). As a C_4 species, switchgrass has high carbon fixation efficiency per unit of radiant energy absorbed. At 0.060 mol CO_2 E^{-1}, the quantum yield (moles of CO_2 absorbed per micromole) is only slightly below that of maize (0.062 mol CO_2 E^{-1}) (Ehleringer and Pearcy, 1983). Additional physiological characterization of switchgrass has been obtained from measurements within a switchgrass germplasm nursery near Stillwater, Oklahoma (Table II). Measurements included characterization of leaf level exchange of CO_2 and H_2O, and seasonal changes in rates of photosynthesis. From such measurements, it was determined that there was high intraspecific variability in physiological characteristics of switchgrass cultivars and both the highest photosynthetic rates and water use efficiencies were clearly associated with highest yields among the 45 varieties tested. Additional traits of switchgrass that are considered by the ALMANAC model (Kiniry et al., 1996) in estimating switchgrass yield potential include canopy bioenergetics and developmental characteristics, nutrient requirements at different growth stages, and contributions to soil quality (soil erosion, soil nutrient status, and soil carbon status). These traits

influence total carbon fixation and production potential, life cycle energetics, production economics, and potential environmental benefits from switchgrass production as a biofuel.

The high investment by switchgrass and other perennial species in root biomass for storage and recovery of nutrients, diverting fixed carbon from harvestable aboveground biomass, represents an important agronomic benefit of these species. It leads to lower requirements for supplemental water and nutrients and hence more stable and cost-effective yields. In addition, the high root turnover in the soil can increase soil organic carbon, which improves soil quality, leading to improved soil and water conservation (Garten and Wullschleger, 2000; McLaughlin and Walsh, 1998). Ultimately, optimization of switchgrass production to balance the mixture of resource use efficiency, total energy and bioproduct recovery, production economics, and fuel quality should be important components of an implementation strategy for utilization of cellulosic crops in bioenergy and bioproduct production.

B.   Parametrization of the ALMANAC Model

ALMANAC is a physiologically based crop production model designed to quantify key plant–environment interactions that influence productivity and resource use by a wide variety of agricultural crops (Kiniry *et al.*, 1992). Parametrization of ALMANAC for estimating switchgrass productivity was based on previous work with Alamo switchgrass at several sites in Texas (Kiniry *et al.*, 1996). Work at locations outside of Texas involved developing appropriate soil parameters to characterize water and nutrient supply potential of each location, and working with the degree days to maturity and the potential leaf area index (LAI) to provide realistic simulations of growth rates and growth dynamics. The model simulates the soil water balance, which requires realistic values for soil depth, soil water–holding capacity, and runoff curve number. Water availability for plant growth is simulated as a function of plant demand, atmospheric demand, rainfall input, and soil water drainage from the upper soil layers. Dry matter production is simulated with light interception using the Beer's law (Monsi and Saeki, 1953) and daily predictions of LAI. A realistic value for potential LAI is needed for each site. Stresses, such as drought or nutrient deficiency, can reduce LAI and biomass in the model.

For analyses in this chapter, ALMANAC was used to check assumptions concerning current and potential yields of switchgrass across regions for which biocellulosic energy supply estimates were of interest. Four representative US locations were selected from a region-wide network of test locations for switchgrass productivity (McLaughlin and Kszos, 2005) with

the idea of using published switchgrass yields to calibrate simulated yields before extending simulations across broader regions. These sites and responsible investigators were Beeville, TX (Bill Occumpaugh), the E.V. Smith site research stations near Tallassee, AL (David Bransby), and eastern Nebraska site near Nickerson, NE (Ken Vogel), and Blacksburg, VA (David Parrish).

Plant parameters used in these simulations included RUE and light extinction coefficient (k) for Beer's Law. The RUE was 4.7 g MJ^{-1} intercepted PAR and the k was -0.33 for all locations. These values were derived from field measurements at Temple, TX (Kiniry *et al.*, 1999). We assumed the potential LAI was near 6.0 for the longer growing season locations and about half as large for the shortest growing season, driest location. Potential LAI was 5.8 for three of the locations and 2.7 for Mead, NE. The base temperature for calculation of growing degree days was 12°C, with an optimum of 25°C (Van Esbroeck, 1996). Values for degree days to maturity were calculated using the actual weather data for the sites. The values for degree days to maturity each year were 2600 in Alabama and Texas, 1400 in Nebraska, and 1100 in Virginia.

C.   Simulated Yields from ALMANAC Versus Actual Yields Within the Region

Working with United States Dept. of Agric.-Natural Resources Conservation Services (USDA-NRCS) soils data and National Oceanic and Atmospheric Administration (NOAA) weather data, we were able to simulate switchgrass yields for these four locations. When compared with available measured Alamo switchgrass yields, ALMANAC yield simulations were found to be in good agreement with yields attained in field test plots at these four locations (Table III).

Table III
Comparison of Simulated Annual Yields of Switchgrass with Actual Yields in Research Plots at Four US Regional Locations

Location	Management	Dry matter yield (Mg ha^{-1} $year^{-1}$)	
		Research plot	ALMANAC simulation
Blacksburg, VA	5-year mean	12.3	12.0
Mead, NE	2-year mean	13.4	13.8
Beeville, TX	1993	11.8	11.7
	1994	11.7	15.7
Tallassee, AL	9-year mean-2 cut	10.5	10.6
	9-year mean-1 cut	10.1	10.2

Switchgrass yield potential was simulated with ALMANAC in this project to both estimate upper yield limits in the field under nonlimiting water and nutrient supply and to provide estimates of time-averaged regional yields that considered both temporal variability in climate and spatial variability in soils within regions. Simulated upper limit yields under field conditions with water and nutrient limitations removed were in the range of 50 Mg ha^{-1} year^{-1} (results not shown), very similar to the scaled maximum plant yield observed in the Tennessee clonal nursery (Table II). Field-scale-yield simulations with ALMANAC, which included both weather-dependent variations over time (13 years) and spatial variability across 27 soil types, have provided estimations of both the level and variability of yields to be expected under typical climatic variability across the study region. Both minimum and average yields within a region shown in Fig. 3 will be important in establishing required acreage to supply a biorefinery plant with adequate feedstock. The higher ratio of minimum to average yield levels associated with the climate and soils in the Southeast and South-Central states suggest that the same land area in those states would provide a more dependable supply of feedstock than in the upper Midwest.

Estimates of water use efficiency (WUE) in switchgrass based on empirical data have also been used with ALMANAC both to explore the adequacy of rainfall across the region to supply moisture demands of high-yielding switchgrass and to compare switchgrass WUE with the demands of maize. In the first instance we calculated the adequacy of rainfall amounts across the study region to supply the moisture demands of a 66% increase in yields estimated to be possible by year 2025 based on breeding studies.
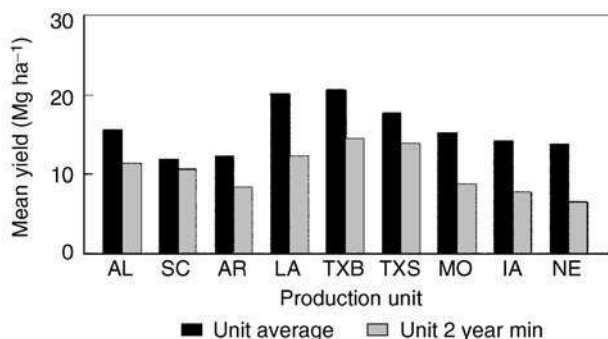
Figure 3 Potential variability in yields will be important to scaling feedstock production areas to maintain a continuous supply to large biorefinery systems. Here the ALMANAC model has been used to interface currently achieved yield levels in test plots with the influences of both soil type and climate variation to express the relative ''risk'' of lower than average feedstock supply capacity for each production unit.

Based on ALMANAC simulations of total crop water use (transpiration and evapotranspiration), moisture levels projected to be available from 13-year-average annual rainfall were adequate to meet the annual demands of switchgrass at projected yield levels (Fig. 4). By plotting projected yields across the region based on current yields, climate, and soil data (Fig. 5), estimates of changes in WUE with increasing yield can be also derived with ALMANAC. Higher values for WUE were clearly associated with higher yields ($P < 0.01$). Thus, we can expect improved WUE to be an offshoot of improved yields through breeding. This may make projected water demand (Fig. 4) somewhat less than we assumed. Such simulations will require
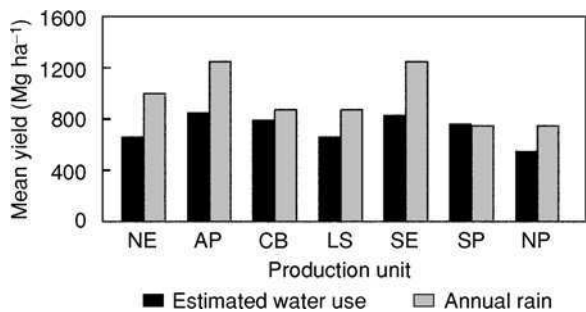
Figure 4 Estimated annual water use from ALMANAC for seven production regions at yield levels projected for 2025 suggests that average annual rainfall (13-year average) will exceed plant requirements in all areas except the US Southern Plains, where studies suggest that intermittent irrigation may strongly increase yields. The POLYSYS regions in the United States are Northeast (NE), Appalachia (AP), Corn Belt (CB), Lake States (LS), Southeast (SE), Southern Plains (SP), and Northern Plains (NP).
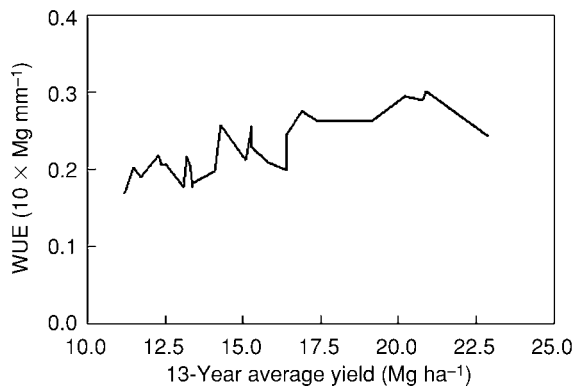
Figure 5 Calculated WUE from 13-year average switchgrass production simulated across 27 soil types by ALMANAC.

additional field data for validation but are compatible with physiological measurements of switchgrass under field conditions in a breeding nursery (Table II).

In a second application of ALMANAC's projections of WUE, we compared the relative water use of switchgrass and maize. In this application we have contrasted total crop water used by both cropping systems across 27 soil types, 13 years, and 9 test regions. Across all regions, the WUE ratio of switchgrass to maize was 40% higher on a biomass production basis and over 300% higher on the basis of biomass production for bioenergy (grain only for maize). We compared WUE calculated both in terms of whole plant yield and on the basis of biomass actually used in energy conversion (Fig. 6). In the primary maize production regions of the Midwest, WUE expressed as total biomass production per unit of water used for maize and switchgrass is highly influenced by soil type, but these WUE values for the two species were similar overall. As one moves outside this region, however, the relative WUE is much higher for switchgrass than for maize. Because the grain is typically the only plant part of maize used for ethanol production and maize grain is only about half of the total aboveground biomass, maize grain WUE is necessarily less favorable relative to switchgrass.
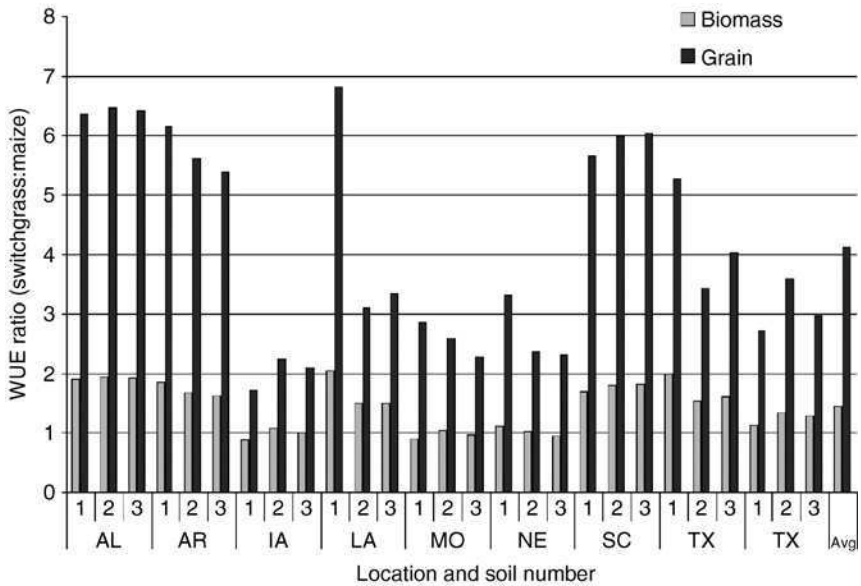
Figure 6 Comparative water use efficiency (WUE) of switchgrass and maize across 3 soil types in each of 9 production regions. WUE values were derived with ALMANAC based on comparative yields and water use of the two crops averaged over 13 y of climatic data for each production region.

Finally, switchgrass yield estimates with ALMANAC have been used in providing important validation of yield assumptions in the region with an econometric model, POLYSYS (Ugarte and Ray, 2000). The POLYSYS model was used to evaluate the potential effects of yield and price of switchgrass on the US agricultural economy as discussed below.

IV. ASSESSING ECONOMIC IMPACTS OF WIDESPREAD DEPLOYMENT OF SWITCHGRASS IN A NATIONAL BIOENERGY PROGRAM

Regional analyses of the economic impacts of widespread utilization of a bioenergy crop have been assessed with POLYSYS, an econometric model developed for evaluating regional crop production economics (Ugarte and Ray, 2000). The POLYSYS model incorporates crop production data across 305 agricultural districts within the eastern two-thirds of the United States for which USDA baseline crop production data are available. The model integrates data on crop yields, production costs, and sale prices to evaluate relative profitability and market penetration. Such information is important for policy analysis but can also reveal important information about relationships among price, yield, demand, and production demographics. The POLYSYS analyses indicated that average yields of a crop like switchgrass, which will likely be grown on marginal lands, will be influenced by the price paid for switchgrass (McLaughlin *et al.*, 2002). Average US switchgrass yield was predicted to decrease from 11 to 9.0 Mg ha^{-1} year^{-1} as the farm gate price increased from \$30.3 Mg^{-1} and 3.1 million ha of supply area to \$52.37 Mg^{-1} and 21 million ha of production (McLaughlin *et al.*, 2002). This effect results from the influence of price on the minimum site quality on which the crop can be profitably grown and, hence, the minimum yield that will be economically viable in the induced supply stream. To project future yields of switchgrass we have assumed that, like maize, a steady rate of yield improvement of base yield levels can be maintained over multiple decades with a sustained and vigorous breeding program. Based on 3–5% per year gain demonstrated for some upland switchgrass varieties in the Midwest (Taliaferro, 2002) and the 7.5% per year gain for lowland varieties realized in a single study in the southeastern United States (Bouton, 2002), we estimated three average rates of improvement of baseline yield levels: one for upland varieties best adapted to the cooler, shorter growing season of the Northern Plains and Lake States (1.5% gain per year above baseline); an intermediate rate of 3% per year for the Cornbelt with intermediate conditions; and the highest rates (5% per year for lowland varieties grown in the southeastern United States). These improvement rates represent the mid to

upper range of improvement for commercial varieties based on past breeding research (McLaughlin and Kszos, 2005).

Projected yields will be heavily dependent on the baseline yields used to calculate them. We used ALMANAC to estimate means and ranges of yields for three soil types within each of nine Agricultural Supply Districts upon which POLYSYS is based. Since POLYSYS yields are estimated field-scale yields based on expert opinion of agronomists within the regions, we penalized the ALMANAC yield estimates for each of nine POLYSYS subregions by 30% to adjust for overestimation of field-scale yields by smaller research plots. These adjusted yields generated with actual soils and 13 years of meteorological data are compared with the regional baseline POLYSYS yields in Table IV. Also included in this table are estimated yield gains for each of the respective regions in response to 10 years (2015) and 20 years (2025) of breeding at gain rates matched to regions as discussed earlier. Averaged annual yields of switchgrass estimated for 27 soil types in nine states by ALMANAC and aggregated into the three test regions in Table IV were 12.22 Mg ha^{-1} year^{-1}, 4% higher than baseline yields in POLYSYS for these same regions. Thus, we considered POLYSYS baseline and projected yields as reasonable estimates of field-scale yields to be expected over larger regions in the United States.

In this study, we used POLYSYS to evaluate the influence of projected yield increases on the availability, price, and economic impacts of producing switchgrass as a feedstock for biorefineries in the RBAEF project. The

Table IV
Estimates of Annual Dry Matter Yield Potential for each of Seven US Production Regions Based on Annual Increase 1.5–5% per Year Over Current Baselines Yields

US production region	Baseline yields (Mg ha^{-1} year^{-1}) Averages (ranges)	Almanac yields (Mg ha^{-1} year^{-1}) Averages[a]	Annual gains (year^{-1}) (%)	Projected future yields (Mg ha^{-1} year^{-1}) 2015	2025
Northeast	10.89 (7.8–12.3)		1.5	12.6	14.20
Appalachia	13.06 (9.8–14.76)		5	19.6	26.2
Corn Belt	13.37 (11.07–15.05)	12.15	3	17.4	21.5
Lake States	10.73 (7.83–13.42)		1.5	12.4	14.0
Southeast	12.28 (7.60–14.42)	10.47	5	18.5	24.6
Southern Plains	9.61 (5.70–13.37)	14.04	5	14.5	19.3
Northern Plains	7.76 (4.47–12.28)		1.5	8.9	10.1

[a]Yields were estimated with the ALMANAC model for six counties in each of three Agricultural Statistical Districts within each of the designated regions. Yields from ALMANAC represent averages of 13 years of simulation with a range of soil types and actual annual meteorological conditions with those regions.

endpoint time periods for estimation in this application were the years 2013 and 2025. The 2013 estimation provided a 10-year maximum forward projection from a 2003 USDA forecasting baseline available at the time these analyses began (i.e., the initiation year was 2004). Yield gains during this time period were at the rate specified in Table IV. The second projection endpoint, 2025, required extension beyond the USDA forecasts. For this application, the USDA forecasting framework was fixed at 2013 levels and dynamic factors were rates of yield gain (constant increase per year from 2013 levels) and prices offered for switchgrass. Agricultural policy changes were restricted to assumptions about allowable export markets (constant or variable) and associated crop prices influenced by supply and demand. Annual rates of yield gain through breeding (Table IV) were considered to extend for a total of 22 years from the 2003 baseline for this application. With the POLYSYS model we combined anticipated rates of yield increase with a range of projected feedstock prices. Outputs from these simulations included total area on which switchgrass would likely be grown based on its relative profitability to farmers compared to conventional crops within each production region; the average yields and total production of switchgrass in these areas; changes in net returns to agriculture from switchgrass and other crops; and finally, reductions in government price support needed as a result of improved prices and farm income.

Results of the POLYSYS simulations (Table V) indicated that introduction of switchgrass as a bioenergy crop could have major and positive implications for the US agriculture. The level of these impacts will be influenced both by yield increases and by the price offered for switchgrass. At a minimum price of $33 Mg^{-1}, over 4.9 million ha are anticipated to be in production after the first 10 years (2013), producing 130 million Mg year^{-1}.

Table V
Switchgrass-Planted Hectares, Production, Average Yield, Change in Net Crop Revenue and Average Annual Savings in Government Payments at Four Price Levels in Years 2013 and 2025 (Under Increasing Exports Baseline Assumption)

Switchgrass price ($ Mg^{-1})	Millions of hectares planted		National production (millions of Mg year^{-1})		Average yield (Mg ha^{-1} year^{-1})		Change in net crop revenue (millions of $)		Average annual savings in government payments (millions of $)	
	2013	2025	2013	2025	2013	2025	2013	2025	2013	2025
33	5.1	7.6	62	130	11.95	17.18	3991	4936	775	1129
44	7.4	11.4	84	182	11.35	15.90	6609	11,982	1647	1661
55	10.6	14.9	118	227	11.12	15.25	13,690	20,587	2217	2011
66	12.9	17.1	143	255	11.10	14.91	24,045	31,492	2419	2135

At \$66 Mg^{-1} these figures more than double in 2013 and more than triple by 2025. Net farm income increases dramatically due to both improved profitability derived from substituting switchgrass for less productive crops as well as improved prices for other crops. The range in this projected effect is from \$3.99 billion at \$33 Mg^{-1} in 2013 to \$32.5 billion at \$66 Mg^{-1} in 2025. Combined with and resulting from improved farm income are substantial reductions in the need for government subsidy payments. The magnitude of these reductions prorated to the quantity of switchgrass produced is on the order of \$8.26 Mg^{-1} of switchgrass produced. Total benefits to agriculture of switchgrass produced under this scenario would be \$123 Mg^{-1} or nearly twice the price offered for switchgrass as a bioenergy feedstock at the \$66 Mg^{-1} level in 2025.

The relative effectiveness of price and yield on farm profitability are of particular interest relative to the emphasis of this paper on breeding potential of switchgrass. Yields attained by 2013 and 2025 in Table V are lower than the projected yields based on breeding progress in Table IV. This is because POLYSY is a dynamic modeling tool that incorporates new and improved seed at the time when demand develops and fields planted to those seed sources remain in production for 10 years before they are planted to newer seed sources that become available later. Thus, the innate yield potential of fields in production in 2013 will be an aggregate of seed sources planted from 1 to 10 years earlier.

Increasing yields through breeding as well as increasing the price offered to farmers for switchgrass can have dramatic effects on total switchgrass production (Fig. 7). Because of the dynamic nature of switchgrass introduction into the marketplace discussed earlier, average switchgrass yields are projected to be only marginally improved by 2013. The geometric average yield across regions in the baseline condition in Table IV was 11.33 Mg ha^{-1} year^{-1}. For this application we reduced this baseline yield by 10% by increasing the cutting height from 10 to 15 cm.[1]

By 2014 the average simulated yield varies between 11.10 and 11.95 Mg ha^{-1} year^{-1}, a 10% increase over baseline. By 2025 the gain is an additional 45% to 15.81 Mg ha^{-1} year^{-1}. Projections of the effects of an aggressive and successful breeding program must consider the time it will take to incorporate genetically improved material into a large-scale production system. Because switchgrass takes 2–3 years to attain maximum yields after planting, reestablishing a switchgrass field with an improved variety will reduce average yields over a 10-year cycle by around 15% from levels that might have been attained at full yield capacity for 10 years.

---

[1]This translates into a baseline yield of 10.20 Mg ha^{-1} year^{-1} after imposing the 10% harvest penalty, resulting from increasing cutting height from 10 to 15 cm in the POLYSYS runs for this application.
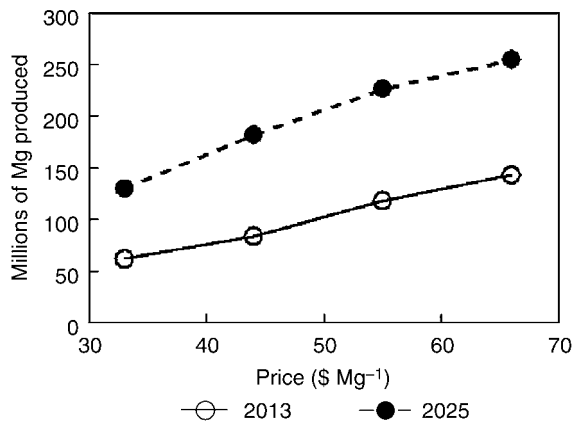
Figure 7 Relationship between price offered to farmers for switchgrass and total switchgrass production at 10 and 20 years after an aggressive breeding program is projected to begin. The increase in total production in 2025 is due to rather modest increase in average yield of 4.48 Mg ha^{-1} year^{-1} (from 11.4 to 15.9 Mg ha^{-1} year^{-1} in 10 years).
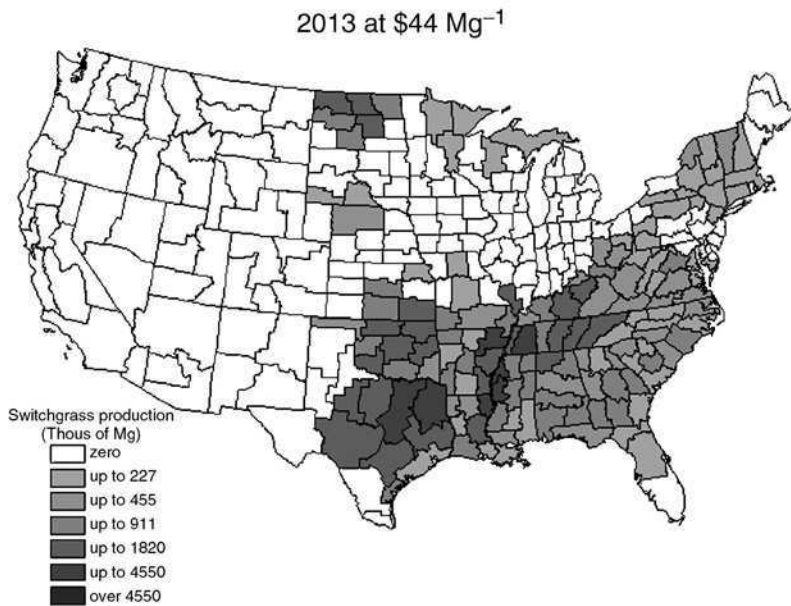
Figure 8 For year 2013, projected switchgrass production density at a delivered price of $44 Mg^{-1} at projected yield levels using POLYSYS stimulations of market penetration. Export levels of other crops are allowed to increase as switchgrass production increases.

2025 at $44 Mg^{-1}
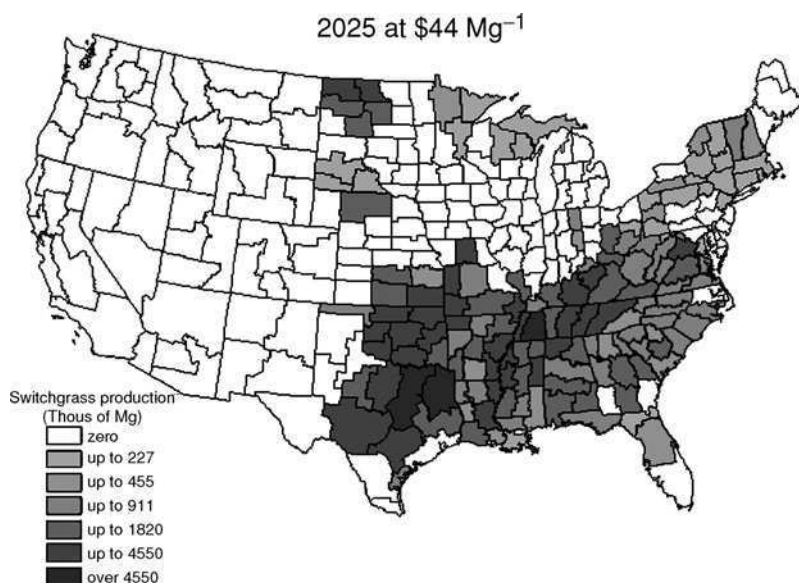
Figure 9 For year 2025, projected switchgrass production density at a delivered price of $44 Mg^{-1} at projected yield levels with yields increased by intensive breeding efforts using POLYSYS simulations of market penetration. Export levels of other crops are allowed to increase as switchgrass production increases.

The other major variable that will influence the rate of development of biofuels supply systems is the regional variability in production potential, a result of both land production capacity and regional economic factors which favor specific crops. Based on regional variability in production potential for the United States at $44 Mg^{-1} in 2013 and 2025 (Figs. 8 and 9), potential productivity within the eastern half for the United States varies from 0 to over 4.5 million Mg within individual POLYSYS production units. By 2013, numerous locations are already projected to be able to supply more than the 1.59 million Mg year^{-1} required to fuel a 4540 Mg day^{-1} biorefinery of the type being proposed by the RBAEF project by 2013. This is based on a modest fuel price of $40 dry Mg^{-1} and relatively small effective increases in yield potential over present levels. In Fig. 10A and B the combined effects of projected yield increases to 2025, increasing the delivered price of switch-grass to $66 Mg^{-1}, and altering agricultural export policy are considered. Combined price and yield increases in 2025 would significantly increase the production density of switchgrass (Fig. 9 vs Fig. 10A) and the total
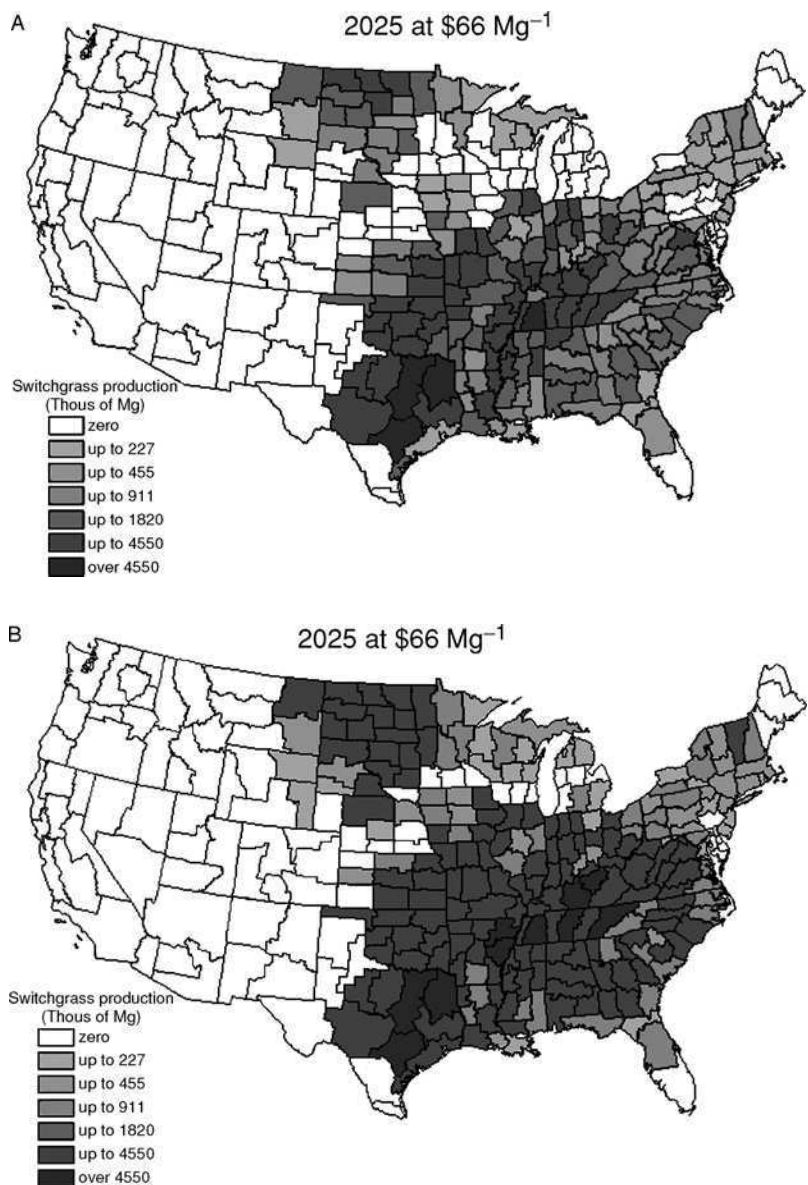
Figure 10   (A) With increasing exports of other crops, POLYSYS projection of regional switchgrass production density with both increasing price (delivered price of $66 Mg^{-1}) at yield levels projected to be obtained by year 2025 by intensive breeding efforts. (B) Identical to Fig. 10A, but with constant exports of other crops.

production by approximately threefolds (see also Fig. 7). In addition, changes in crop export limits posed on conventional crops with which switchgrass would compete for land can also influence switchgrass production levels. The POLYSYS simulations indicate that with unlimited export of these crops as assumed in Figs. 8, 9, and 10A, prices of these crops will rise as the land base on which they are grown shrinks and demand increases. As a consequence, conventional crops would become more competitive with switchgrass. By limiting exports of these crops, prices of these crops would be lower and switchgrass would become relatively more competitive. Thus, a flat export policy further increases the competitive potential and production density of switchgrass (Fig. 10B).

Feedstock supply will be a vital component of efforts to achieve increased energy self-sufficiency on a national scale. Such systems will ultimately require utilization of multiple feedstocks to maximize US bioenergy and bioproduct potential. Dedicated feedstocks, such as switchgrass, should play an important supply-stabilizing role in such systems, and an aggressive breeding program building on past progress will be important in developing full production capacity. Recognition of the high efficiency of switchgrass in production of bioenergy per unit of input of both energy and water should be important considerations in maximizing energy production potential. Early comparisons of energy budgets of switchgrass and maize (McLaughlin and Walsh, 1998) indicated that energy gains from producing and converting switchgrass bioenergy to ethanol energy will be large (over threefold) for switchgrass. The input energy to produce switchgrass is only about 8% of the output energy in the biomass. With projected yield improvements described here, we expect this input energy to decrease even further. In addition, the favorable WUE of switchgrass should make it increasingly attractive in maximizing energy output efficiency on a landscape level. In the shorter term, strategic location of initial plants in regions with high innate production potential using the best available varieties should allow earlier initial deployment of commercial scale plants and testing of economically viable feedstock supply systems.

V.   CONCLUSIONS

Similarities in the physiology and early breeding success between maize and switchgrass indicate that an aggressive breeding program similar to that of maize could lead to a doubling of yield of the best lowland varieties in 20–30 years to around 22 Mg ha^{-1} year^{-1} on areas of high production potential. The ALMANAC model and the POLYSYS model were used to make regional forecasts of the increase in switchgrass in regions having

conventional agricultural markets. These models simulated the total dry tonnage of switchgrass production, the increase in farm income, and reductions in the level of government subsidies needed. Within 10 years of initiating intensive breeding efforts, even at relatively lower switchgrass prices of $44 Mg^{-1}, significant increases in farm income ($6.6 billion) and government subsidy reductions ($1.6 billion) are projected at yields only 10% higher than current capacity. Significant opportunities would exist for locating the first 4540 Mg day^{-1} biorefinery even at this early stage. After 20 years of yield improvement it is estimated that, at a price of $66 Mg^{-1}, 254 million Mg of switchgrass would be produced on more than 17 million ha of cropland on which switchgrass would be more profitable than conventional crops. This production is projected to increase net farm income by $31 billion and reduce the need for government subsidies by $21 billion. An aggressive switchgrass breeding program using modern breeding techniques, including molecular biology, would provide significant economic gains to the nation as it searches for avenues of greater energy self-sufficiency. The lag time for incorporating the best new varieties into perennial grass agriculture systems dictates that such efforts should be initiated very early in the planning cycle to maximize their effects.

Finally, the coupling of research results in basic physiology, breeding, yield management, and modeling production demographics and economics described here has been most useful in feeding national energy policy analysis in the RBAEF project. The results have contributed significantly to RBAEF analyses and recommendations, several of which were included in the Energy Policy Act (2005), and these results have important implications for future agronomic research and development. On the one hand they suggest that a large fraction of this nation's transportation energy requirements could be met with bioenergy feedstock production on currently managed lands with little or no additional land requirements. On the other hand they suggest that it can be very useful to address significant policy issues by coupling basic agronomic research with linked simulation models at multiple scales.

REFERENCES

Adams, W. E., and Stelly, M. (1958). A comparison of Coastal and common bermudagrass (*Cynodon dactylon* (L) Pers.) in the Piedmont Region: I. Yield response to fertilization. *Agron. J.* **50**, 457–459.

Bouton, J. H. (2002). Bioenergy crop breeding and production research in the southeast. Final Report for 1996–2001. ORNL/SUB-02–19XSV810C/01, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Burton, G. W. (1947). Breeding bermuda grass for the southeastern United States. *J. Am. Soc. Agron.* **39**, 551–569.

Burton, G. W. (1954). Coastal bermuda grass. Bull. N.S. 2. GA Agric. Exp. Stn., Athens.

Burton, G. W. (1982). Improved recurrent restricted phenotypic selection increases bahiagrass forage yields. *Crop Sci.* **22**, 1058–1061.

Burton, G. W. (1985). Spaced-plant-population-progress test. *Crop Sci.* **25**, 63–65.

Burton, G. W. (1992). Recurrent restricted phenotypic selection. *Plant Breed. Rev.* **9**, 101–113.

Burton, G. W., and Mullinix, B. G. (1998). Yield distributions of spaced plants within Pensacola bahiagrass populations developed by recurrent restricted phenotypic selection. *Crop Sci.* **38**, 333–336.

Carreker, J., Adams, W. E., Jackson, W. A., and Dawson, R. N. (1972). Management factors affecting Coastal bermudagrass yield and water use. *J. Soil Water Conserv.* **27**, 14–17.

Casler, M. D. (2001). Breeding forage crops for increased nutritional value. *Adv. Agron.* **71**, 51–107.

Casler, M. D., Vogel, K. P., Balasko, J. A., Berdahl, J. D., Miller, D. A., Hansen, J. L., and Fritz, J. O. (2000). Genetic progress from 50 years of smooth bromegrass breeding. *Crop Sci.* **40**, 13–22.

Conger, B. V., Denchev, P. D., and Alexandrova, K. S. (1996). Development of *in vitro* systems for switchgrass (*Panicum virgatum*). 1995 Annual Report to ORNL, Bioenergy Feedstock Development Program.

Duvick, D. N. (1997). What is yield? *In* "Developing Drought Tolerant and Low N-Tolerant Maize" (G. O. Edmeades, M. Banziger, H. R. Mickelson, and C. B. Peña-Valdivia, Eds.), pp. 332–335. Proc. Symp, March 25–29, CIMMYT, El Batan, Mexico.

Duvick, D. N. (1999). Heterosis: Feeding people and protecting natural resources. *In* "Heterosis in Crops," Chapter 3, pp. 19–29. Proc. Intern. Symp. Genetics and Exploitation of Heterosis in Crops.

Dwyer, L. M., Tollenaar, M., and Stewart, D. W. (1991). Changes in plant density dependence of leaf photosynthesis of maize (*Zea mays L.*) hybrids, 1959 to 1988. *Can. J. Plant Sci.* **71**, 1–11.

Dwyer, L. M., Stewart, D. W., and Tollenaar, M. (1992). Analysis of maize leaf photosynthesis under drought stress. *Can. J. Plant Sci.* **72**, 477–481.

Earl, H., and Tollenaar, M. (1998). Differences among commercial maize (*Zea mays* L.) hybrids in respiration rates of mature leaves. *Field Crops Res.* **59**, 9–19.

Echarte, L., and Andrade, F. H. (2003). Harvest index stability of Argentinean maize hybrids released between 1965 and 1993. *Field Crops Res.* **82**, 1–12.

Energy Policy Act. (2005). *In* Public Law 109-58, passed by the U.S. Congress on July 29, 2005.

Ehleringer, J. R., and Pearcy, R. W. (1983). Variation in quantum yields for CO_2 uptake among C_3 and C_4 plants. *Plant Physiol.* **73**, 555–559.

Fasoulas, A. C., and Fasoula, V. A. (1995). Honeycomb selection designs. *Plant Breed. Rev.* **13**, 87–139.

Fasoulas, A. C., and Fasoula, V. A. (2000). Honeycomb breeding: Principles and applications. *Plant Breed. Rev.* **18**, 177–250.

Fehr, W. R. (1987). "Principles of Cultivar Development." Macmillan, New York.

Fischer, R. A., and Evans, L. T. (1999). Yield potential: Its definition, measurement, and significance. *Crop Sci.* **39**, 1544–1551.

Fischer, R. A., Rees, D., Sayre, K. D., Lu, Z.-M, Condon, A. G., and Saavedra, A. L. (1998). Wheat yields associated with higher stomatal conductance and photosynthetic rates and cooler canopies. *Crop. Sci.* **38**, 1467–1475.

Garten, C. T., Jr., and Wullschleger, S. D. (2000). Soil carbon dynamics beneath switchgrass as indicated by stable isotope analysis. *J. Environ. Qual.* **29**, 1–9.

Goodman, M. M., and Brown, W. L. (1988). Races of corn. *In* "Corn and Corn Improvement" (G. F. Sprague and J. W. Dudley, Eds.), pp. 33–79. American Society of Agronomy, Inc., Madison, Wisc..

Greene, N. (2004). Growing Energy. How Biofuels Can Help End America's Oil Dependence. (Natural Resources Defense Council available at http://www.nrdc.org/air/energy/biofuels/contents.asp).

Hopkins, A. A., Vogel, K. P., and Moore, K. J. (1993). Predicted and realized gains from selection for *in vitro* dry matter digestibility and forage yield in switchgrass. *Crop Sci.* **33**, 253–258.

Kiniry, J. R., Williams, J. R., Gassman, P. W., and Debaeke, P. (1992). A general, process-oriented model for two competing plant species. *Trans. ASAE.* **35**, 801–810.

Kiniry, J. R., Sanderson, M. A., Williams, J. R., Tischler, C. R., Hussey, M. A., Ocumpaugh, W. R., Read, J. R., Van Esbroek, G. A., and Reed, R. L. (1996). Simulating Alamo switchgrass with the ALMANAC model. *Agron. J.* **88**, 602–606.

Kiniry, J. R., Tischler, C. R., and Van Esbroek, G. A. (1999). Radiation use efficiency and leaf CO_2 exchange for diverse C_4 grasses. *Biomass and Bioenergy* **17**, 95–112.

Kiniry, J. R., Xie, Y., and Gerik, T. J. (2002). Similarity of maize seed number responses for a diverse set of sites. *Agronomie* **22**, 265–272.

Kiniry, J. R., Bean, B., Xie, Y., and Chen, Pei-yu. (2004). Maize yield potential: Processes and simulation modeling in a high-yielding environment. *Agric. Systems* **82**, 45–56.

Lindquist, J. L., Arkebauer, T. J., Walters, D. T., Cassman, K. G., and Dobermann, A. (2005). Maize radiation use efficiency under optimal growth conditions. *Agron. J.* **97**, 72–78.

Loomis, R. S., and Amthor, J. S. (1996). Limits of yield revisited. *In* "Increasing Yield Potential in Wheat: Breaking the Barriers" (P. Reynolds, S. Rajaram, and A. McNab, Eds.), pp. 76–89. Mexico, DF, CIMMYT.

Loomis, R. S., and Amthor, J. S. (1999). Yield potential, plant assimilatory capacity, and metabolic efficiencies. *Crop Sci.* **39**, 1584–1596.

McKay, A. D., and Barber, S. A. (1986). Effect of nitrogen on root growth of two corn genotypes in the field. *Agron. J.* **78**, 699–703.

McLaughlin, S. B., Bouton, J. H., Bransby, D. I., Conger, B. V., Ocumpaugh, W. R., Parrish, D. J., Taliaferro, C. M., Vogel, K. P., and Wullschleger, S. D. (1999). Developing switch-grass as a bioenergy crop. *In* "Proceedings of the Fourth National New Crops Symposium" (J. J. Janick, Ed.), pp. 282–299. ASHS Press, Alexandria, VA.

McLaughlin, S. B., De La Torre Ugarte, D. G., Garten, C. T., Jr., Lynd, L. R., Sanderson, M. A., Tolbert, V. R., and Wolf, D. D. (2002). High-value renewable energy from prairie grasses. *Environ. Sci. Technol.* **36**, 2122–2129.

McLaughlin, S. B., and Kszos, L. A. (2005). Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass and Bioenergy* **28**, 515–535.

McLaughlin, S. B., and Walsh, M. (1998). Evaluating environmental consequences of producing herbaceous crops for bioenergy. *Biomass and Bioenergy* **10**, 1–8.

Monsi, M., and Saeki, T. (1953). Über den lichtfaktor in den pflanzengesellschaften und seine bedeutung fur die stoffproduktion. *Jpn. J. Bot.* **14**, 22–52.

Newell, L. C., and Eberhart, S. A. (1961). Clone and progeny evaluation in the improvement of switchgrass, *Panicum virgatum* L. *Crop Sci.* **1**, 117–121.

Parrish, D. J., Wolf, D. D., Fike, J. H., and Daniels, W. L. (2003). Switchgrass as a biofuels crop for the upper southeast: Variety trials and cultural improvements. Final report for 1997 to 2001. ORNL/SUB-03–19SY163C/01. Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Pietsch, D., Synatschk, L., Bertran, J., and Foyt, F., III (1999). Corn performance tests in Texas. Technical Report 99-25. Texas Agricultural Experiment Station, College Station.

Pimmentel, D., Doughty, R., Carothers, S., Lamberson, S., Bora, N., and Lee, K. (2002). Energy inputs in crop production: Comparison of developed and developing countries. *In* "Food Security and Environmental Quality in the Developing World" (R. Lal, D. Hansen, N. Uphoff, and S. Slack, Eds.), pp. 129–151. CRC Press, Boca Raton, FL.

Rajcan, I., and Tollenaar, M. (1999). Source:sink ratio and leaf senscence in maize: I. Dry matter accumulation and partitioning during grain filling. *Field Crops Res.* **60**, 245–253.

Shapouri, H., Duffield, J. A., and Graboski, M. S. (1995). Estimating the Net Energy Balance of Corn ethanol. Agric. Econ. Report No. 721 US Dept. of Agric., Econ. Res. Service, Office of Energy.

Sladden, S. E., Bransby, D. L., and Aiken, G. E. (1991). Biomass yield, composition, and production costs for eight switchgrass varieties in Alabama. *Biomass and Bioenergy* **1**, 119–122.

Steinauer, E. M., and Collins, S. L. (1996). Prairie ecology-the tallgrass prairie. *In* "Prairie Conservation" (R. B. Samson and F. L. Knopf, Eds.), pp. 39–52. Island Press, Washington, DC.

Talbert, L. E., Timothy, D. H., Burns, J. C., Rawlings, J. O., and Moll, R. H. (1983). Estimates of genetic parameters in switchgrass. *Crop Sci.* **23**, 725–728.

Taliaferro, C. M. (2002). Breeding and selection of new switchgrass varieties for increased biomass production. ORNL Technical Report ORNL/SUB-02–19XSY162C/01.

Taliaferro, C. M., Vogel, K. P., Bouton, J. H., McLaughlin, S. B., and Tuskan, G. A. (1999). Reproductive characteristics and breeding improvement potential of switchgrass. *In* "Proceedings of the 4th Biomass Conference of the Americas Biomass, A Growth Opportunity in Green Energy and Value-Added Products" (R. Overend and E. Chornet, Eds.), pp. 147–153. Elsevier Sciences, Oxford, United Kingdom.

Tokatlidis, I. S. (2001). The effect of improved potential yield per plant on crop yield potential and optimum plant density in maize hybrids. *J. Agric. Sci.* **137**, 299–305.

Tollenaar, M. (1983). Potential vegetative productivity in Canada. *Can. J. Plant Sci.* **63**, 1–10.

Tollenaar, M. (1989). Genetic improvement in grain yield of commercial maize hybrids grown in Ontario from 1959 to 1988. *Crop Sci.* **29**, 1365–1371.

Tollenaar, M. (1992). Is low plant density a stress in maize? *Maydica* **37**, 305–311.

Tollenaar, M., and Aguilera, A. (1992). Radiation use efficiency of an old and a new maize hybrid. *Agron. J.* **84**, 536–541.

Tollenaar, M., McCullough, D. E., and Dwyer, L. M. (1994). Physiological basis of the genetic improvement of corn. *In* "Genetic Improvement of Field Crops" (G. A. Shafer, Ed.), pp. 183–236. Marcel Dekker, New York.

Tollenaar, M., and Wu, J. (1999). Yield improvement in temperate maize is attributable to greater stress tolerance. *Crop. Sci.* **39**, 1597–1604.

Tollenaar, M., and Lee, E. A. (2002). Yield potential, yield stability, and stress tolerance in maize. *Field Crops Res.* **75**, 161–169.

Ugarte, D. G. D., and Ray, D. E. (2000). Biomass and bioenergy applications of the POLYSYS modeling framework. *Biomass and Bioenergy* **18**, 291–308.

Van Esbroeck, G. A. (1996). Genetic control of switchgrass growth and development. (Ph.D. Dissertation). Texas A&M University.

Vogel, K. P., Dewald, C. I., Gorz, H. J., and Haskins, F. A. (1985). Development of switchgrass, indiangrass, and eastern gamagrass: Current status and future range improvement in Western North America. Proceedings Range Management, Salt Lake City, Utah, February 14, 1985, pp. 51–62.

Vogel, K. P., Haskins, F. A., and Gorz, H. J. (1981). Divergent selection for *in vitro* dry matter digestibility in switchgrass. *Crop Sci.* **21**, 39–41.

Vogel, K. P. (2000). Improving warm-season grasses using selection, breeding, and biotechnology. *In* "Native Warm-Season Grasses: Research Trends and Issues" (K. J. Moore and B. Anderson, Eds.), pp. 83–106. CSSA and ASA. CSSA Special Publ. 30, Madison, WI.

Vogel, K. P. (2004). Switchgrass. *In* "Warm-Season Grasses" (L. E. Moser, L. Sollenberger, and B. Burson, Eds.), pp. 561–588. ASA-CSSA-SSSA Monograph, Madison, WI.

Wilkins, P. W., and Humphreys, M. O. (2003). Progress in breeding perennial forage grasses for temperate agriculture. *J. Agric. Sci.* **140**, 129–150.

Wullschleger, S. D., Sanderson, M. A., McLaughlin, S. B., Biradir, D. P., and Rayburn, A. L. (1996a). Photosynthetic rates and ploidy levels among populations of switchgrass. *Crop Sci.* **36**, 306–312.

Wullschleger, S. D., Gunter, L. E., and Garten, C. T. (1996b). Genetic diversity and long-term stability of yield in the bioenergy crop switchgrass. Five-year summary report to ORNL Biofuels Feedstock Development Program.

This page intentionally left blank

Index